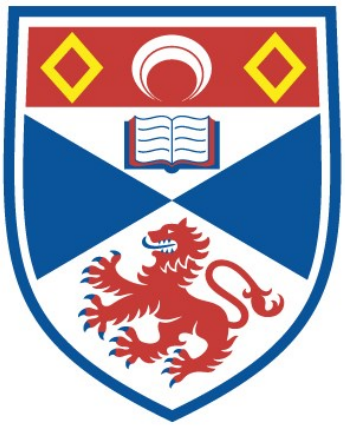
REINITIATION OF MEIOSIS IN POLYCHAETE (ANNELIDA) OOCYTES

Lesley Ann Paterson

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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REINITIATION OF MEIOSIS IN POLYCHAETE (ANNELIDA) OOCYTES

by Lesley Ann Paterson

Submitted for the Degree of Doctor of Philosophy in the

University of St Andrews

School of Biology

Division of Environmental & Evolutionary Biology

Gatty Marine Laboratory

University of St Andrews

May 1999

To Mum, Dad, Jacks and Stew.

Declaration

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Abstract

This thesis presents ultrastructural and biochemical information on meiotic reinitiation during oocyte maturation in the polychaetes, *Arenicola marina*, *A. defodiens* and *Nereis virens*.

The ultrastructural changes during meiotic maturation was characterised in the oocytes of *Arenicola marina* and *Nereis virens* using transmission electron microscopy In addition to germinal vesicle breakdown, release of the prophase I block was signified by major cortical changes in both species. The ultrastructure of fertilization in *A. marina* was independent of whether the oocytes were matured *in vivo* and spawned or matured *in vitro* by CMF.

Oocyte maturation in *Arenicola marina* is controlled by a hormonal cascade that is initiated by the prostomial maturation hormone, PMH, and followed by the coelomic maturation factor, CMF (Watson and Bentley, 1997). Results presented here demonstrated that PMH has a molecular mass greater than 10 kDa, yet how this molecule triggers CMF activity remains unknown.

M-phase promoting factor (MPF) consists of two subunits, cdk1 and cyclin B, and is responsible for the control of mitosis and meiosis. The cytoplasmic "second messenger" that transduces the hormone signal to the activation of MPF in the oocyte cytoplasm was investigated in the two *Arenicola* species and is discussed. MPF regulation was investigated in *Arenicola marina* and *Nereis virens* oocytes. MPF activation was driven by the dephosphorylation of cdk1 and phosphorylation of cyclin B. The results indicate that as with all other higher eukaryotes, the precursor of MPF in *A. marina* oocytes was maintained inactive by the phosphorylation of threonine 14 and tyrosine 15 (or equivalent residues) on the cdk1 subunit. In contrast to other organisms, however, only a fraction of the cdk1 present was complexed to cyclin B and utilised during meiotic reinitiation. All the cdk1 in *N. virens* oocytes was joined with cyclin B but results suggest that the inactive complex contained tyrosine-only phosphorylated cdk1.

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Abbreviations

1-MeAde	1-methyladenine
5-HT	5- hydroxytryptamine (serotonin)
17α,20β-DP	17α, 20β-dihydroxy-4-pregnen-one
ATP	adenosine triphosphate
CAK	cdk activating kinase
cAMP	adenosine 3',5'-cyclic monophosphate
cdk	cyclin-dependent kinase
cdk1	cyclin dependent kinase 1
CLSM	confocal laser scanning microscopy
CMF	coelomic maturation factor
DAG	diaglycerol
dbcAMP	dibutryl cAMP
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethyleneglycol-bis(b-aminoethylether) N,N,N',N'tetraacetic acid
FSH	follicle stimulating hormone
FSW	filtered seawater
G-proteins	guanine nucleotide binding regulatory proteins
GR	grid reference

GSH	gonad stimulating hormone
GTH	gonadotropin
GVBD	germinal vesicle breakdown
HDP	hormone dependent period
HETE	hydroxyeicosatetraenoic acid
IGF-1	insulin growth factor 1
IP_3	inositol triphosphate
kDa	kilo dalton
LH	lutenizing hormone
MAF	microwave assisted fixation
MAP kinase	mitogen activated protein kinase
MAP kinase kinase	mitogen activated protein kinase kinase
MIF	meiosis inducing factor
MPF	M-phase promoting factor
PAGE	polyacrylamide gel electrophoresis
PKA	protein kinase A (cAMP-dependent protein kinase)
PKC	protein kinase C (Ca++/phospholipid/DAG-dependent kinases)
PMH	prostomial maturation hormone
SMF	sperm maturation factor (8, 11, 14-eicosatrienoic acid)
TEM	transmission electron microscopy

TFSW	twice filtered seawater
Thr 14	threonine 14
Tyr 15	tyrosine 15

Chapter 1

Introduction to the Regulation of Oocyte Maturation

1.1 Mitosis and Meiosis

The division of a cell to produce two identical daughter cells is termed "mitosis", and is the method by which both tissue growth and repair is achieved. Mitosis can also produce a new individual, in the case of asexual reproduction (e.g. fission and budding). However, this results in the formation of a clone (all individuals genetically identical). Sexual reproduction, in contrast, generates genetic variability and involves the fusion of two specialist reproductive cells during fertilization. These reproductive cells, termed "gametes", are produced by meiosis, the generation of cells with half the parent number of chromosomes.

Cell division is a cyclical process, of which the meiotic and mitotic "M-phase" form only one part (see Nurse, 1994, for review). Interphase is prior to M-phase and divided into three stages, G_1, S and G_2. (Fig. 1.1). Following the termination of one cell cycle, the cell enters G_1, the first gap, and this is followed by S-phase, during which the cell synthesises and replicates DNA ready for the next division. The second gap, G_2, follows before the cell enters the meiotic or mitotic M-phase. M-phase is signified by the separation and division of the chromosomes, giving rise ultimately into new cells by cytokinesis (see Wolf *et al.*, 1999, for review)

Mitosis consists of a single round of DNA replication followed by a single division to produce two daughter cells containing the identical chromosome number as the parent cell (see Koshland and Strunlikv, 1996, for review). Meiosis, however, is characterised by two successive divisions to produce gametes (see Dekel, 1995; Cobb and Handel, 1998, for review). Mitosis, although a continual process, is divided into several stages for convenience: prophase, metaphase, anaphase and telophase (see Fig. 1.2 for further details). Meiosis is also divided into separate stages, beginning at

prophase I with a nucleus containing replicated DNA. At metaphase I, each pair of homologous chromosomes lines up along the spindle. The separation of the chromosome pairs begins at anaphase I, and is completed by telophase I. During the second meiotic division, the 2 sister chromatids of each chromosome become separated and segregated to form 4 daughter cells, each with half the parent chromosome number. Spermatogenic production is achieved by meiosis as described above (see Cobb and Handel, 1998; Hecht, 1998, for review), oocyte production (see Masui and Clarke, 1979, for review) however, follows a slightly different pattern (see Fig. 1.3 for further details).

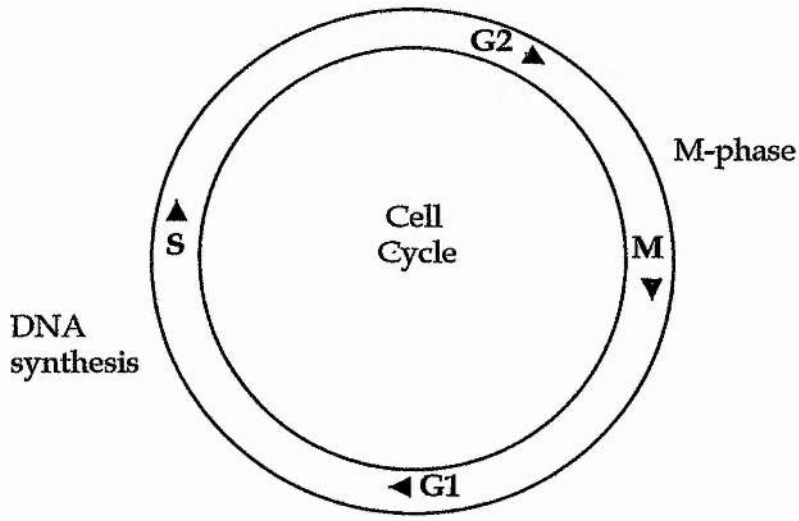


Figure 1.1: Diagrammatic Representation of the Cell Division Cycle

1.2 Oocyte Maturation

Oogenesis is the term given for the process of oocyte growth and development (for examples see Wourms, 1987; Wickramsinghe and Albertini, 1993; Nagahama *et al.*, 1995). Oocytes are produced within an ovary or ovotestis where they may, or may not, remain until the completion

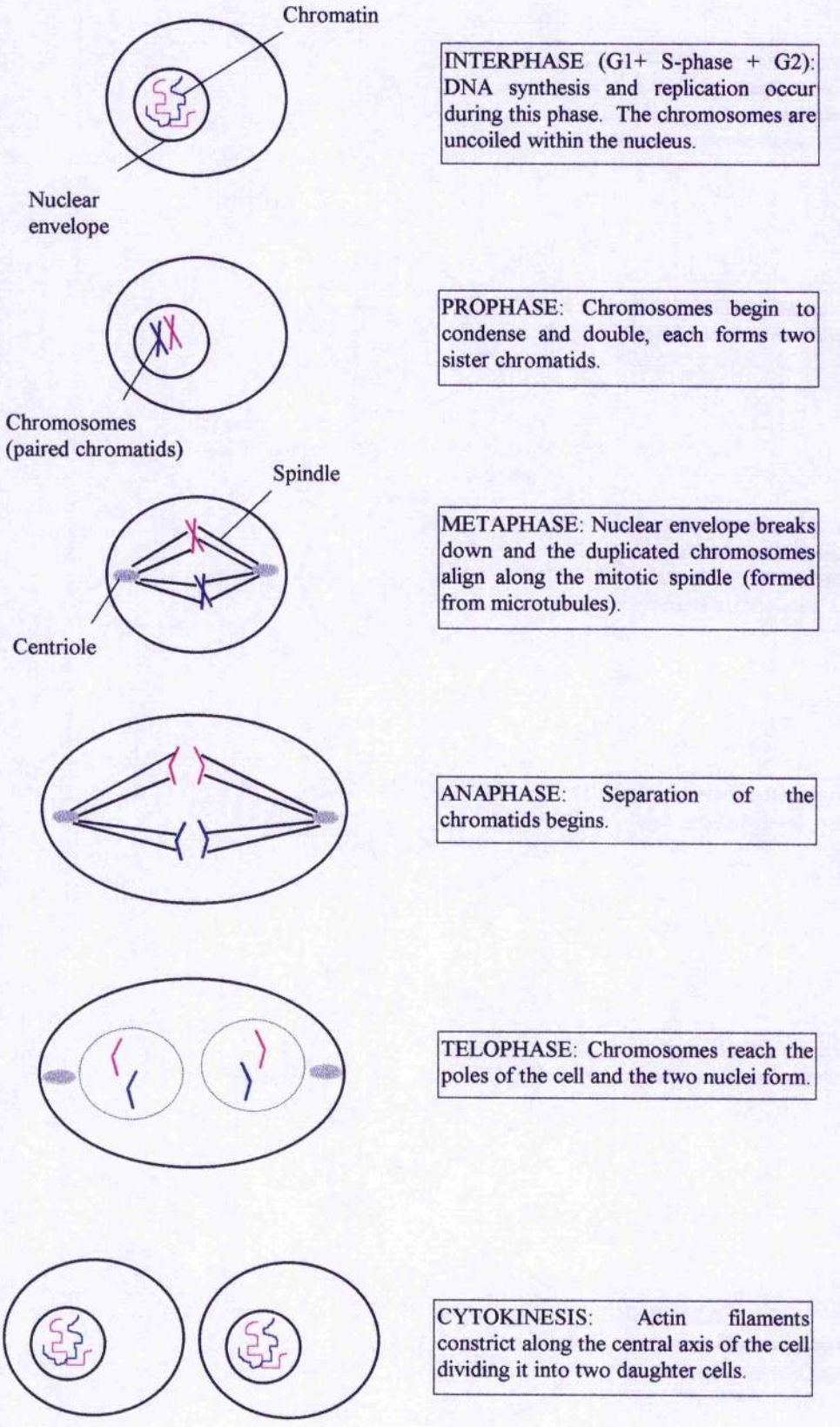
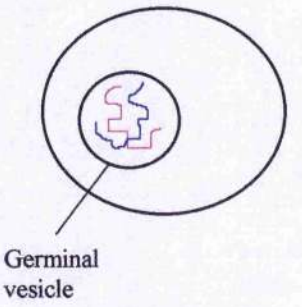
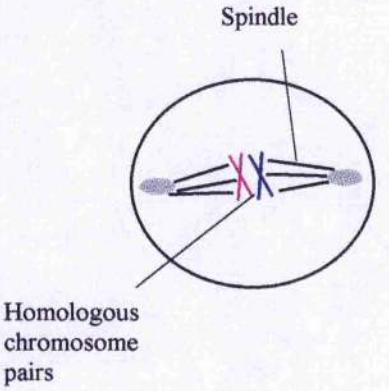


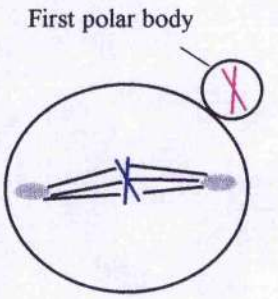
Figure 1.2. Mitosis



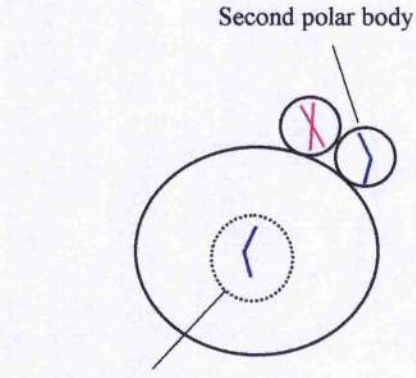
G2/M BORDER: Oocyte is arrested at the border between G2 and prophase I. The large nucleus is termed a germinal vesicle and contains replicated DNA.



METAPHASE I: Homologous chromosomes pair up and align on the spindle.



FIRST POLAR BODY: Homologous chromosome pairs are separated. One set is packaged up in membrane, becomes extruded outside the oocyte, and is called the first polar body. The remaining set of chromosomes align upon the metaphase plate at METAPHASE II. The sister chromatids then become separated.



SECOND POLAR BODY: Following separation of the sister chromatids, only one set is used to form the female pronucleus. The surplus set of chromatids is incorporated into the second polar body. The oocyte has completed meiosis (now termed a mature egg) and contains the female pronucleus, with half the chromosome number as the parent cell.

Figure 1.3. Oocyte Maturation

of oogenesis. In addition to various synthetic processes (e.g. vitellogenesis) during oogenesis, each oocyte enters into meiosis to prepare for future fertilization. Prior to the completion of meiosis, the fully grown oocytes are arrested at prophase I (see Masui and Clarke, 1979, for review). The transition from prophase I arrest to female pronuclear formation is referred to as "oocyte maturation" (Wilson, 1925). Once the prophase I block has been lifted, meiosis either continues to pronuclear formation or undergoes a second arrest, which is species dependent. In the majority of animals, the prophase I block is removed following exposure to the maturation inducing factor (MIF), which is generally of hormonal origin, before meiosis arrests once more (see sections 1.2.2 and 1.2.3). This block is released on insemination, and maturation is then completed (see section 1.2.2 and 1.2.3). In other animals, insemination alone releases the prophase I block (see section 1.2.1) or oocyte maturation proceeds to completion without suspension, and fertilization occurs at the pronuclear stage (see section 1.2.4). The stage at which fertilization occurs has led to the categorisation of oocytes into 4 groups (Rothschild, 1956):

- Class I: oocytes are inseminated at prophase I of meiosis (the germinal vesicle still intact)
- Class II: oocytes are inseminated at the metaphase I of meiosis (the first spindle has formed)
- Class III: oocytes are inseminated at metaphase II of meiosis (the second spindle has been constructed and the first polar body has formed)
- Class IV: oocytes are inseminated following the completion of meiosis (both polar bodies have been produced and the pronucleus has formed)

The resumption of meiosis is driven by the activation of an intracellular enzyme called the M-phase promoting factor or MPF (see section 1.5) and is the intracellular factor responsible for driving the oocyte from interphase to M-phase (see section 1.5). In this introduction, oocytes have been referred to as "prophase I arrested". However, at the end of oogenesis, oocytes are technically arrested during the transition from G_2 to prophase I, hence at the G_2/M-phase border, but here they shall be referred to as "prophase I arrested" for simplicity.

The sections below describe oocyte maturation throughout the animal kingdom. The first part introduces the MIFs (the "first messengers"), which act outside the oocyte to trigger maturation. The second part is a review of the work completed on the "second messenger" which is the intracellular factor responsible for relaying the MIF signal to the cytoplasm and leads to the activation of MPF.

1.2.1 Oocytes Fertilized at the Germinal Vesicle Stage (Class I)

In molluscs, the entire process of oogenesis occurs in the ovary or ovotestis, and each of the oocytes are associated with follicle cells (Bottke, 1974) In the majority of mollusc species, 5-HT (5-hydroxytryptamine also known as "serotonin") is the neurohormone that induces maturation (see section 1.2.2). However, in the surf clam *Spisula solidissima,* injection of 5-HT into the ovaries results in spawning, yet the germinal vesicles remain intact (Hirai *et al.*, 1988). These oocytes are not released from their prophase I block until insemination (Hirai *et al.*, 1988).

Induction of oocyte maturation by insemination is also observed in a number of polychaetes such as the nereids (Heilbrunn and Wilbur, 1937; Bass and Brafield, 1972; Dorresteijn, 1990). In these species, the oocytes undergo extra-ovarian development as solitary freely floating cells within the

coelomic cavity of females (Brafield and Chapman, 1967). There is no terminal nuclear maturation of the oocytes, and they are spawned with intact germinal vesicles until meiosis is reinitiated in the seawater following insemination (Heilbrunn and Wilbur, 1937; Bass and Brafield, 1972; Dorresteijn, 1990). The oocytes of the polychaete *Pomatoceros triqueter* (Cragg, 1939; ap Gwynn and Jones, 1971) and the echiuroid worm *Urechis caupo* (Gould-Somero and Holland, 1975) are also spawned and fertilized at prophase I.

1.2.2 Oocytes Fertilized at Metaphase I (Class II)

Injection of 5-HT into a number of mollusc species induces oocyte maturation and subsequent shedding (Hirai *et al.*, 1988; Ram *et al.*, 1993; Fong *et al.*, 1994; Désilets *et al.*, 1995). These spawned oocytes are arrested at metaphase of meiosis I until fertilization occurs (Guerrier *et al.*, 1993; Fong *et al.*, 1994; Désilets *et al.*, 1995). 5-HT application *in vitro* will also induce meiotic maturation of mollusc oocytes (Hirai *et al.*, 1988). In addition, Krantic *et al.* (1993) used radio-ligand assays on partially purified oocyte membranes to show that both plasma membrane and vitelline envelope fractions contain binding sites specific for 5-HT. These results together suggest that 5-HT is the native inducer for meiotic maturation in molluscs.

In addition to the Mollusca, oocytes of many Polychaeta are also arrested at metaphase I. In *Arenicola marina* and *A. defodiens* a prostomial (i.e. from the brain) maturation hormone, PMH, is released (Watson *et al.*, 1998). In the former species, this results in the production of a second hormone, the coelomic maturation factor (CMF) which acts upon the oocyte to induce the prophase to metaphase I transition (Watson and Bentley, 1997). In the latter species, however, PMH acts directly on the oocyte to induce maturation (Watson *et al.*, 1998). In the polychaete *Pectinaria gouldii*, a substance from the sub-oesophageal gland has been shown to induce oocyte maturation

(Tweedell, 1980). When the female *P. gouldii* are treated with extracts of these tissues, germinal vesicle breakdown (GVBD), progression to metaphase I and spawning occurs (Tweedell, 1980). In the oocytes of the two polychaetes *Chaetopterus pergamentaceus* and *Sabellaria alveolata*, the prophase I to metaphase I transition is also triggered by extracellular signals. The oocytes of *S. alveolata* are induced to mature by a digestive enzyme that triggers GVBD by proteolysis (Peaucellier, 1977). In the polychaete *C. pergamentaceus* the trigger is an unidentified biochemical in seawater (Ikegami *et al.*, 1976).

1.2.3 Oocytes Fertilized at Metaphase II (Class III)

The African clawed toad *Xenopus laevis* is recognised as the model species for cell cycle control studies in amphibian oocytes. As with other vertebrates, oocyte maturation is stimulated by gonadotropins (GTH). GTH stimulates the synthesis of the steroid hormone, progesterone, which is produced by the follicle cells (Fortune *et al.*, 1975) during ovulation *in vivo* (Masui and Shibuya, 1987) and can also induce maturation *in vitro* (Sadler and Maller, 1983). The site of progesterone action appears to be at specialist receptors on the plasma membrane (Smith and Ecker, 1971). Once the prophase block has been lifted, the oocyte proceeds to metaphase of meiosis II, at which stage development is arrested once again until fertilization.

In teleost fish, oocytes are surrounded by a double layer of follicle cells, the inner granulosa and the outer thecal layer. GTH is synthesised in the pituitary gland of fish under the stimulating action of the gonadotropin-releasing hormone (Nagahama, 1987; Degani *et al.*, 1997). Follicle enclosed oocytes will mature *in vitro* when subjected to GTHs, naked oocytes, however, do not, because the hormone acts upon the follicle cells, which in turn produce the MIF. The search for the true identity of teleost MIF has focused largely on the salmonids. Salmonid ovaries are a particularly

useful model for investigation, as the follicles within are very large, undergo synchronous development and the two follicle cell layers can be separated easily (Nagahama *et al.*, 1995). Results from many studies show that 17α, 20β-dihydroxy-4-pregnen-one (17α,20β-DP) is the principal MIF in salmonid fishes (Nagahama *et al.*, 1995). 17α,20β-DP is synthesised from its inactive precursor form, 17α-hydroxy-progesterone (King *et al.*, 1995; Ohta *et al.*, 1997). It then binds to the oocyte surface at specific sites, which induces maturation and subsequent ovulation of the metaphase II-arrested oocytes (Nagahama *et al.*, 1995). With respect to the non-salmonid fishes, results imply that 17α, 20β-DP is also the MIF in selected species (Haider and Rao, 1992) yet in others, 17α, 20β, 21-trihydroxy-4-pregnen-3-one (20β-hydro-11-deoxycortisol) has been identified as the naturally occurring MIF (Trant and Thomas, 1989).

Much of the work upon the control of mammalian oocyte maturation has been completed upon rodents (primarily mouse) and farm animals (primarily pigs). Mammalian oocytes are surrounded by follicle cells (cumulus) and the transition from prophase to metaphase II is regulated by hormones (see below).

Many studies have focused upon the precise control of mammalian oocyte maturation that is driven by GTHs (see Mattioli, 1996, for review). Two of which are the follicle stimulating hormone (FSH), which regulates oocyte growth, and the lutenizing hormone (LH) which triggers MIF production in the follicle cells. In addition to LH and FSH, other hormones shown to have a potential role are the epidermal growth factor (EGF) and insulin-like-growth factor-I (IGF1) (Reed *et al.*, 1993). Results show that the presence of FSH, LH, EGF or IGF1 enhances oocyte maturation (both nuclear and cytoplasmic) in mammals (Mattioli *et al.*, 1991; Singh *et al.*, 1993; Ding and Foxcroft, 1994; Xia *et al.*, 1994; Sirotkin *et al.*, 1998; Wang *et al.*, 1998).

Fully grown mammalian oocytes will undergo spontaneous maturation when isolated from their follicle cells, indicating that meiotic reinitiation is regulated also by inhibition *via* the surrounding cumulus (Petr *et al.*, 1991).

1.2.4 Oocytes Fertilized at the Pronuclear Stage (Class IV)

Starfish have to date provided the model for oocyte maturation studies of marine invertebrates (Meijer and Guerrier, 1984; Meijer and Mordret, 1994). The oocytes of these organisms are held within the ovary, each being surrounded by single layer of squamous follicle cells (Schroeder and Stricker, 1983). The oocytes are arrested at prophase I of meiosis until maturation is initiated and meiosis then continues without suspension until formation of the female pronucleus. Fertilization, however, usually occurs prior to completion of meiosis, after GVBD (Miyake and Hirai, 1979). Hence starfish are not strictly members of "class IV". Starfish oocyte maturation is a two step hormonal process and the first hormone, a 2 kDa peptide neurohormone (Kanatani and Shirai, 1971) called the gonad stimulating hormone (GSH), is released from the radial nerve (Shirai *et al.*, 1986). The action of GSH induces the follicle cells to release a second hormone, 1-methyladenine (1-MeAde), that acts directly upon the oocyte to stimulate maturation (Kanatani and Shirai, 1967). 1-MeAde was the first marine invertebrate hormone to be identified chemically (Kanatani, 1969; Kanatani *et al.*, 1969). Binding sites to this hormone are located on the surface of the oocyte on both the vitelline envelope and the oocyte plasma membrane (Yoshikuni *et al.*, 1988). Oocytes must be incubated with 1-MeAde for a certain period of time, which is temperature dependent, called the hormone dependent period (HDP), after which exposure is no longer required and maturation will proceed to completion (Guerrier and Dorée, 1975). 1-MeAde is thought to be synthesised as required and evidence for this is provided by Mita *et al.* (1996) using high performance liquid chromatography to show

that high concentrations of ATP are present in the follicle cells. These results indicate that the ATP stores are the substrate for 1-MeAde biosynthesis.

The oocytes of sea urchins are different from the vast majority of organisms, whereby meiosis proceeds until completion, without arrest. The mature eggs (i.e produced two polar bodies and formed a female pronucleus) are spawned, which can be induced under laboratory conditions by injection of potassium chloride into the coelomic cavity (for examples see Rakow and Shen, 1990; Stricker *et al.*, 1992) and then fertilized. Much of the work upon sea urchins has been completed with respect to fertilization biology, particulary calcium waves (see Shen, 1995 for review). As the main topic of this review is concerned with the reinitation of meiosis and not mitosis, which occurs with the sea urchin egg, the work on this species shall not be discussed further.

1.3 The Second Messenger for Oocyte Maturation

The second messenger is the intracellular signal linking MIF/receptor action to the activation of MPF. This section focuses upon the signal transduction pathway involved during release of the prophase I block by hormone induced meiotic maturation. For details of the signal cascade that induces meiotic reinitiation following insemination, refer to chapter 5 (section 5.1).

1.3.1 G-proteins

Guanine nucleotide binding regulatory proteins (G-proteins) are a class of receptors found in the plasma membrane, which transduce signals from extracellular hormones. The involvement of G-proteins at oocyte maturation during signal transduction from MIF receptors has been demonstrated in several organisms, although much of the work has been

performed on starfish. Evidence for the participation of these receptors in starfish oocyte maturation was provided initially by Shilling *et al.* (1989) using a pertussis toxin known to inhibit the action of specific G-proteins. In the presence of this toxin, oocytes will not undergo GVBD, however, micro-injection of the toxin has no effect upon maturation indicating that it is acting at the oocyte surface (Shilling *et al.*, 1989). Further studies (Hoshi *et al.*, 1992; Tadenuma *et al.*, 1992; Jaffe *et al.*, 1993) confirmed that 1-MeAde acts upon membrane receptors upon the oocyte surface and are bound to G-proteins.

G-proteins have also been shown to play a role in oocyte maturation of teleost fish. Results suggest that the alpha subunit of a G-protein is involved in the signal transduction of 17α,20β-DP in the medaka (*Oryzias latipes*) oocyte (Oba *et al.*, 1997). In addition, studies by Gobet *et al.* (1994) provide evidence that the 5-HT receptors found upon mollusc oocytes are also coupled to G-proteins.

In all species investigated so far, evidence shows that G-proteins are coupled to the MIF receptor. However, the precise target of the G-proteins following activation has yet to be determined. The potential signals that may be involved are discussed in the following sections.

1.3.2 Calcium Ions

The role of calcium ions during oocyte maturation has been investigated in many organisms. Several methods are used for exploring the function of these cations during cellular processes. These include observing the effects of calcium effectors, measuring the amounts of calcium using radioactive Ca^{45} and visualising calcium within the cell using intracellular dyes (for examples see Picard *et al.*, 1985a; Lefevre *et al.*, 1995; Duesbery and Masui, 1996).

Early work has provided some evidence that calcium is the

relaying signal between 1-MeAde and the activation of MPF in echinoderms. Following 1-MeAde application to starfish oocytes, a transient increase in cytosolic calcium occurs (Moreau *et al.*, 1978). This rise in calcium, however, is not consistent and furthermore, is neither necessary nor sufficient to induce meiotic reinitiation (Kikuyama and Hiramoto, 1991; Stricker *et al.*, 1994). In addition, the injection of inositol trisphosphate (IP_3) into starfish oocytes, which induces an increase in cellular calcium, fails to release the block at prophase (Picard *et al.*, 1985b). Overall, these results demonstrate that calcium is not the second messenger in mediating the signal from 1-MeAde to MPF, although the possibility of it acting in a secondary role within the pathway cannot be discarded.

Results indicate generally that calcium is involved during the transduction of the 5-HT signal to the activation of MPF in molluscan oocytes (Guerrier *et al.*, 1981; Dubé *et al.*, 1987; Krantic *et al.*, 1991; Abdelmajid *et al.*, 1993a; Guerrier *et al.*, 1993; Gobet *et al.*, 1995) except in the oocytes of the oyster *Crassostrea gigas* (Kyozuka *et al.*, 1997). The work of Krantic *et al.* (1991) shows that 5-HT induces the uptake of calcium, and is ineffective in calcium free seawater. Thus indicating first that calcium is required for meiotic reinitiation and secondly that it is derived from the surrounding seawater. Potassium chloride (KCl) has often been tested for its effects on oocyte maturation because an excess of this chemical induces membrane depolarisation resulting in an influx of calcium from the extracellular environment. Molluscan oocyte maturation is also promoted in excess KCl (Guerrier *et al.*, 1981) and prevented by the use of verapamil, an inhibitor of L-type voltage gated calcium-channels (Kadam *et al.*, 1990). Furthermore, micro-injection of the calcium effector inositol trisphosphate (IP_3) causes oocyte maturation in molluscs (Bloom *et al.*, 1988) and hence the calcium ions may also be derived from intracellular stores.

In polychaetes, some work has been completed upon the potential role of calcium as the second messenger. In *Chaetopterus pergamentaceus,* induction of oocyte maturation is dependent upon the presence of calcium in the external medium (Ikegami *et al.,* 1976) and meiotic maturation can be stimulated in *Arenicola defodiens* prophase arrested oocytes by calcium-modifying drugs (Meijer, 1980). Oocytes of *Sabellaria alveolata* can undergo maturation in seawater lacking divalent cations but will mature by the addition of calcium ionophore A23187 which releases intracellular calcium stores (Peaucellier, 1977).

There are conflicting reports on the role of calcium during oocyte maturation in vertebrates. Results of an earlier study show that meiotic maturation does not occur in response to the micro-injection of IP_3 into the *Xenopus* oocyte (Picard *et al.,* 1985a). Studies completed 10 years later, however, provide evidence that IP_3-induced calcium release does indeed have a function in *Xenopus* oocyte maturation (Han and Lee, 1995). Duesbery and Masui (1996) examined Ca^{45} levels and also studied the effects of calcium chelating buffers in *Xenopus* and demonstrated that release of intracellular calcium stores is required for oocyte maturation, but alone is not sufficient to induce it. Lefevre, *et al.* (1995) used confocal laser scanning microscopy (CLSM) to examine changes in intracellular calcium of mouse oocytes and reported that calcium oscillations occur in these oocytes during maturation. However, Tombes *et al.* (1992) had previousley concluded that mouse oocyte maturation is calcium independent.

1.3.3 Hydrogen Ions

Changes in hydrogen ions have been detected during oocyte maturation in several species (Johnson and Epel, 1982; Cicirelli *et al.,* 1983; Flament *et al.,* 1996; Kyozuka *et al.,* 1997) but changes in pH have only been shown to be sufficient to induce oocyte maturation in certain molluscs

species (Guerrier *et al.*, 1981; Catalan and Yamamoto, 1993; Gobet *et al.*, 1995). Although the role of pH as the second messenger in *Xenopus* has been ruled out (Cicirelli *et al.*, 1983), work by Flament *et al.* (1996) demonstrated that alkalinisation of the cytoplasm is potentially involved in germinal vesicle migration that occurs during progesterone induced oocyte maturation.

1.3.4 Protein Kinase C

The protein kinase C (PKC) family consists of a heterogenous group of related kinases, that differ in their co-factors but have like-catalytic sites (see Nishizuka, 1992; Dekker and Parker, 1994, for review). These kinases regulate other cellular components by the phosphorylation of serine and/or threonine residues (Nishizuka, 1992; Dekker and Parker, 1994). PKCs can be dependent upon calcium ions, and/or stimulated by diaglycerol (DAG) or phospholipids (see Nishizuka, 1992; Dekker and Parker, 1994 for review). They are therefore also known as the Ca^{++}/phospholipid/DAG - dependent kinases.

PKCs initiate meiotic maturation in the oocytes of a range of species and these include starfish (Xu *et al.*, 1993), the polychaete *Chaetopterus pergamentaceus* (Eckberg *et al.*, 1996), the mollusc *Spisula solidissima* (Dubé *et al.*, 1987), the amphibian *Xenopus laevis* (Kwon and Lee, 1991) and rats (Aberdam and Dekel, 1985). In *Xenopus* oocytes, however, PKC activation does not induce meiotic resumption *in vivo* (Hille *et al.*, 1996) but may in fact negatively regulate oocyte maturation, as a decrease in its activity stimulates progression to metaphase II (Varnold and Smith, 1990). Recent papers, however, describe a positive effect of PKC in the initiation of oocyte maturation in starfish (Stapleton *et al.*, 1998) and *C. pergamentaceus* (Eckberg *et al.*, 1996). Evidence from both these papers strongly supports the hypothesis that PKC is a candidate for the second messenger during *in vivo* transduction of the signal from MIF to MPF activation. Moreover, results

suggest that PKC is not only the second messenger but activates MPF directly in *C. pergamentaceus* oocytes (Eckberg *et al.*, 1996). In starfish, evidence shows that cdc25 is the direct activator of MPF (see section 1.5.1), but results suggest that PKC is a part of the signal transduction pathway leading to MPF activation, possibly by activating cdc25 itself (Stapleton *et al.*, 1998).

1.3.5 Adenosine 3',5'-cyclic monophosphate (cAMP)

In starfish oocytes, one of the earliest biochemical changes noted during oocyte maturation is the decrease in cAMP (Meijer and Zarutskie, 1987). Chemical effectors of cAMP concentration can induce or prevent oocyte maturation in starfish oocytes, due to decreased or increased cAMP concentration, respectively (Karaseva and Khotimchenko, 1991). These results have led to the hypothesis that meiotic prophase arrest is maintained through high cAMP levels within the oocyte. Nevertheless, although reduced cAMP levels lead to conditions that will now permit maturation to occur they are not sufficient to induce meiotic reinitiation alone (Meijer *et al.*, 1989b).

The potential role of cAMP in the preservation of meiotic prophase arrest and signal transduction of the MIF signal is also important in vertebrates. Following MIF treatment, the oocytes of amphibians (Speaker and Butcher, 1977) and catfish (Haider and Chaube, 1995) show a significant decrease in cAMP levels. In agreement with these findings, when cAMP levels are kept at a high level, amphibian oocyte maturation (Schorderet-Slatkine and Baulieu, 1982) and fish oocyte maturation (DeManno and Goetz, 1987) fails to occur. Furthermore, cAMP antagonists induce GVBD in mammalian prophase I arrested oocytes (Bornslaeger *et al.*, 1986; Eppig, 1991; Jung *et al.*, 1992) and increases in cAMP inhibit GVBD (Homa, 1988; Petr *et al.*, 1991; Mattioli *et al.*, 1994). However, to the contrary, the

oocytes of some mammalian species exhibit a transient rise in cAMP levels at the initial stages of meiotic maturation (Mattioli *et al.*, 1994).

Collectively, the studies described above indicate strongly that the high levels of cAMP in prophase oocytes prevents meiotic progression in a wide range of organisms. The questions remain, however, about how cAMP levels within the oocytes are controlled, and what are the intracellular effects induced by the decrease in cAMP. The action of cAMP during oocyte maturation is thought to involve adenylate cyclase and protein kinase A (PKA). Adenylate cyclase triggers the production of cAMP, and activators of this enzyme (e.g. using forskolin) induce an intracellular rise in cAMP levels and inhibit maturation in amphibian oocytes (Kwon and Lee, 1991), fish oocytes (DeManno and Goetz, 1987; Finet *et al.*, 1988; Haider and Chaube, 1995) and bovine oocytes (Homa, 1988). Furthermore, 1-MeAde exposure to starfish oocytes inhibits the activity of adenylate cyclase which in turn induces a decrease in cAMP concentration (Karaseva *et al.*, 1996). PKA is a cAMP-dependent protein kinase and a decrease in its activity induces meiotic maturation in mouse oocytes (Bornslaeger, *et al.*, 1986), *Xenopus* oocytes (Huchon *et al.*, 1981; Matten *et al.*, 1994) and starfish oocytes (Dorée *et al.*, 1981).

Overall, the role of cAMP, adenylate cyclase and PKA was elegantly summarised by (Hille *et al.*, 1996) for *Xenopus* oocytes, but this could be equally applicable to all organisms: Interaction with a G-protein leads to a decrease in the level of adenylate cyclase, resulting in a decrease in the concentration of cAMP, which in turn leads to a reduction in the cAMP-dependent protein kinase (PKA). Presumably PKA is phosphorylating some factor, possibly another kinase, whose activity maintains meiotic arrest. Alternatively, PKA may phosphorylate a kinase, thereby inhibiting its activity which would otherwise lead to meiotic maturation. In vertebrates,

data gathered from various authors show that the function of PKA is to prevent the activation of the Mos kinase. Mos is discussed in the following section, alongside MAP (Mitogen Activated Protein) kinase.

1.3.6 Mos and MAP Kinase

The proto-oncogene product Mos is a serine-threonine protein kinase found in vertebrate oocytes. Mos synthesis is a pre-requisite for *Xenopus* oocyte maturation and during the prophase I block it is negatively regulated by the PKA activity in *Xenopus* oocytes (Daar *et al.*, 1993; Matten *et al.*, 1994; Matten *et al.*, 1996). In mammals, the role of Mos at the G_2 to M-phase transition is less clear, and results indicate that this gene product has no function until the metaphase II block (Hashimoto, 1996). The existence of Mos within the oocytes of invertebrate species has not been demonstrated.

MAP kinases are a family of phosphorylating proteins of a molecular weight around 45 kDa. They are activated by extracellular signals such as hormones, growth factors and neurotransmitters. MAP kinase is activated in frog oocytes by its MAP kinase kinase (Kosako *et al.*, 1994), which in turn is activated by Mos. The precise function of MAP kinase in *Xenopus* has yet to be clarified but evidence is provided that it partakes in the activation of MPF (Kosako *et al.*, 1994; Gotoh and Nishida, 1995; Huang and Ferrell, 1996) and/or is responsible for spindle assembly (Takenaka *et al.*, 1997). In other organisms, MAP kinase has received less attention and the role of this enzyme remains obscure. Like *Xenopus* (Nebreda and Hunt, 1993), MAP kinase is activated before or simultaneously with MPF activation in bovine oocytes (Fissore *et al.*, 1996) and hence could have a role in MPF activation. In mouse (Gavin *et al.*, 1994) and goat (Dedieu *et al.*, 1996) oocytes, however, MAP kinase is activated after MPF and hence cannot therefore have a role in its activation. Overall, functions of Mos and MAP kinase during meiotic maturation in vertebrates may not be ubiquitous in different species.

Although Mos does not exist, or at least, has not been identified in the oocytes of marine invertebrates, MAP kinase does. Exposure to the MIF results in the activation of MAP kinase in mollusc oocytes (Abdelmajid *et al.*, 1994) starfish oocytes (Sadler and Ruderman, 1998) and polychaete oocytes (Eckberg, 1997). In the latter two cases, results demonstrate that MAP kinase activation is not, however, required for oocyte maturation (Eckberg, 1997; Sadler and Ruderman, 1998).

1.3.7 Other Potential Second Messengers

Other substances with a potential role during signal transduction from MIF to MPF are the hydroxyeicosatetraenoic acids (HETEs) and proteases. Arachidonic acid induces oocyte maturation in starfish, due to the formation of active metabolites (Meijer *et al.*, 1986a, b). Of the arachidonic acid metabolites tested, only 8(R)-HETE shows maturation inducing activity and its potential role in oocyte maturation signal transduction is discussed by Meijer *et al.* (1984). Interestingly, 8(R)-HETE will also induce a decrease in cAMP concentration (Meijer *et al.*, 1986a). More recently Varaksin *et al.* (1992) has shown that both arachidonic acid and its metabolites can also stimulate GVBD in the *Spisula solidissima* oocyte.

Another potential signal in the second messenger pathway is protease activity. Takagi Sawada *et al.* (1992) reported that the activity of a 650 kDa proteosome increases in starfish oocytes shortly after 1-MeAde application. Furthermore, an earlier publication (Takagi Sawada *et al.*, 1989) showed that protease inhibitors (e.g. leupeptin) will inhibit MPF production in 1-MeAde treated oocytes, supporting the hypothesis that a trypsin-like protease is involved in hormone action.

1.3.8 Summary

In many species, the MIF binds to a receptor located upon the

oocyte surface, which is coupled to a G-protein. The second messenger has yet to be confirmed, but there is the likelihood of more than one signal involved in the transduction pathway, because different chemicals have been found which modulate and enhance maturation but alone are not sufficient to induce it. It is evident that in many animals cAMP has an important role to play in the maintenance and release of the prophase block.

1.4 M-PHASE PROMOTING FACTOR

During mitosis and meiosis, the cell undergoes the sudden dramatic structural changes of chromosome condensation, cytoskeletal reorganisation and nuclear envelope breakdown. What drives such fundamental and dramatic reorganisation of the cell during division? Historically, the cell cycle has been studied primarily by investigating the DNA (e.g. synthesis, replication and monitoring of chromosome movement). However, in the last twenty years studies have become focused upon the underlying control mechanisms and asked how is the cell division cycle regulated and controlled. This has led to the discovery of a family of protein kinases that are responsible for cell cycle control (see Dorée and Galas, 1994, for review). The most well studied member of this family is the M-phase promoting factor (MPF), that regulates entry into meiotic or mitotic M-phase in all eukaryotic cells. The discovery of MPF was due to two, essentially independent, lines of investigation: the study of oocyte maturation (principally in amphibians and echinoderms) and yeast genetics.

1.4.1 Discovery of MPF

Evidence for a cytoplasmic-derived maturation inducing factor originated from work upon the oocytes of two species of toad, *Bufo bufo* and *B. viridis* (Dettlaff *et al.*, 1964). Dettlaff *et al.* (1964) discovered that

injection of cytoplasm from maturing toad oocytes into prophase-arrested toad oocytes, resulted in GVBD.

In the early 1970s, three papers were published concerning meiotic and mitotic cell division (Johnson and Rao, 1970; Masui and Markert, 1971; Smith and Ecker, 1971). Johnson and Rao (1970) worked upon HeLa cells and discovered that chromosome condensation is induced in interphase cells, when fused with mitotically active cells. The other two studies were directed towards oocyte maturation in the frog *Rana pipiens*: Smith and Ecker (1971) demonstrated that progesterone is effective only if applied externally to the oocyte surface and will not induce GVBD if injected within the oocyte. Moreover, Masui and Markert (1971) inserted cytoplasm, removed from maturing frog oocytes, into immature oocytes, and discovered that GVBD and maturation occurred without the application of progesterone. The results of these three studies prompted each to suggest the presence of a cytoplasmic factor within the cell that promotes M-phase, which was named the "maturation promoting factor" by Masui and Markert (1971), the "intracellular inducer" by Smith and Ecker (1971) and "mitotic inducer" by Johnson and Rao (1970).

Also at this time, and following the identification of 1-MeAde (Kanatani, 1969; Kanatani *et al.*, 1969), research into the control of starfish oocyte maturation received much attention. 1-MeAde was found to be effective if applied externally but maturation did not proceed if this chemical was micro-injected intracellulary (Kanatani and Hiramoto, 1970). Furthermore, injection of cytoplasm from maturing starfish oocytes into prophase arrested oocytes induced meiotic reinitiation (Kishimoto and Kanatani, 1976). These results demonstrated that, as with amphibian oocytes, there was evidence of a maturation promoting factor (MPF) that initiates release of the prophase I block in starfish oocytes.

Parallel to the studies on oocyte maturation, Nurse and his co-workers (for example see Nurse, 1975) were working upon the fission yeast, *Schizosaccharomyces pombe* to identify, clone and characterise the regulatory genes responsible for mitotic cell cycle control. Yeast can be either haploid or diploid, and the use of the haploid form is advantageous when conducting genetic experiments, as this eliminates the possibility of having more than one allele. Nurse, therefore, used haploid yeast strains mutated at one allele, to determine the effect of that gene upon the cell cycle. Nurse used temperature sensitive mutants of yeast. The yeast colonies could, therefore, be placed outside their restrictive temperature (the mutated gene could, for example, prevent cell division) and the allele functions normally again, allowing propagation of the strain for further investigations. Such a mutant produced interesting results that were reported in the mid-seventies (Nurse *et al.*, 1976). The protein product of this gene had kinase activity (phosphorylated other proteins) and was required for entry into mitosis and named cdc2 (after Cell Division Cycle).

1.4.2 Ubiquity of cdc2 and MPF

Both lines of investigation (yeast genetics and oocyte maturation) continued their studies independently through the late 1970s and early 1980s. With respect to yeast genetics, a number of other cell cycle control genes were cloned (see Kohli, 1987, for review). In addition, homologues to the $cdc2^1$ gene in fission yeast were discovered in the budding yeast *Saccharomyces cerevisiae* ($CDC28^1$, Beach *et al.*, 1982) and humans ($CDC2^1$, Draetta *et al.*, 1987; Lee and Nurse, 1987). All three gene homologues were found to be structurally similar (Hindley and Phear, 1984; Lee and Nurse, 1987) and geneticists found that the gene CDC28 of the budding yeast *S. cerevisiae*, could functionally cross complement cdc2 of fission yeast *S. pombe* (Beach *et al.*, 1982). Hence, as the cdc2 gene was homologus in both yeast and humans, it was likely that homologues to the cdc2 gene were present in

all eukaryotic organisms.

Further work continued on MPF using starfish and amphibian oocyte maturation as models. In addition, more and more cells were found to contain MPF activity: mammalian cultured cells (Sunkara *et al.*, 1979); mouse oocytes (Hashimoto and Kishimoto, 1988); surf clam oocytes (Kishimoto *et al.*, 1984); slime mould (Adlakha *et al.*, 1988); mitotically cleaving embryos of *Xenopus* and starfish (Kishimoto *et al.*, 1982b; Gerhart *et al.*, 1984). MPF from different cell-types was found to induce division in each other: starfish oocyte MPF was not species specific (Kishimoto and Kanatani, 1977); mouse or surf clam oocyte MPF could induce maturation in immature starfish oocytes (Kishimoto *et al.*, 1984); amphibian oocyte MPF could induce starfish oocyte maturation (Kishimoto *et al.*, 1982a). Moreover, cytoplasm from mitotically active cells could induce the reinitiation of meiosis in oocytes (Sunkara *et al.*, 1979; Kishimoto *et al.*, 1982a; Adlakha *et al.*, 1988) and *vice versa* (Lohka and Maller, 1985). Together these results demonstrated that MPF was functionally active irrespective of species or phylum, in both invertebrates and vertebrates, and was not confined within the bounds of controlling and initiating oocyte maturation but was responsible for the regulation of both meiosis and mitosis.

1.4.3 Union between MPF and cdc2

In 1988 and 1989 several important studies were published, describing the common denominator between MPF and $p34^{cdc2}$ (the protein product of the yeast cdc2 gene). It was found that MPF consisted of 2 major polypeptides in the oocytes of *Xenopus* (Dunphy *et al.*, 1988; Gautier *et al.*, 1988; Lohka *et al.*, 1988) and starfish (Labbé *et al.*, 1989a). The smallest polypeptide had an apparent molecular weight of 32 kDa in *Xenopus* oocytes

[1] Different cases were used in naming the gene homologues to differentiate from one another, i.e. CDC2 referred to the gene homologue from humans and cdc2 in yeast.

(Gautier *et al.*, 1988; Lohka *et al.*, 1988) and 34 kDa in starfish oocytes (Arion *et al.*, 1988; Labbé *et al.*, 1988; Labbé *et al.*, 1989a; Labbé *et al.*, 1989b). In addition, it was found that antibodies directed against $p34^{cdc2}$ cross-reacted with the 32 kDa protein in *Xenopus* MPF (Gautier *et al.*, 1988) and the 34 kDa protein in starfish MPF (Arion *et al.*, 1988; Labbé *et al.*, 1988) Due to the similarity in molecular weight ($p34^{cdc2}$=34 kDa, *Xenopus* MPF subunit = 32kDa, starfish MPF subunit = 34 kDa), their parallel functions (i.e. regulation of cell division) and the fact that the yeast $p34^{cdc2}$ antibodies were able to bind with the MPF subunits, it was concluded that the 32 kDa subunit of *Xenopus* and 34 kDa subunit of starfish were analagous to the cdc2 gene product in fission yeast, $p34^{cdc2}$ (Arion *et al.*, 1988; Gautier *et al.*, 1988; Labbé *et al.*, 1988; Labbé *et al.*, 1989a; Labbé *et al.*, 1989b) So the link between the yeast cell division cycle regulatory gene, cdc2 and MPF was discovered.

1.4.4 Cyclin B

In 1989, purification revealed that cyclin B was the second subunit of MPF (Labbé *et al.*, 1989a). Cyclin B belongs to a family of proteins, first recognised in developing surf clam embryos (Rosenthal *et al.*, 1980), maturing starfish oocytes (Rosenthal *et al.*, 1982) and developing sea urchin oocytes (Evans *et al.*, 1983). They were called cyclins due to their cyclical appearance in the cell as they are periodically synthesised and broken down in conjunction with the cell cycle (Evans *et al.*, 1983). Evans (1983) stated that cyclins were connected to the cell division cycle, but no direct evidence was provided until later. Pines and Hunt (1987) described how synthetic sea urchin mRNAs for cyclins, injected into prophase *Xenopus* oocytes, resulted in meiotic resumption.

Labbé *et al.*, (1989a) concluded that MPF was a heterodimeric protein complex consisting of one molecule of $p34^{cdc2}$ and one molecule of cyclin B. MPF has now been confirmed as the universal and fundamental regulator

for the onset of M-phase. MPF, previously known as the "Maturation Promoting Factor" was consequently re-named by John Gerhardt as MPF, the " M-phase promoting factor" (Kishimoto, 1996).

1.5 REGULATION OF MPF

$p34^{cdc2}$ was so called for many years, however, present terminology has renamed it cyclin dependent kinase 1, cdk1. In all eukaryotic organisms, active MPF consists of cdk1 complexed to cyclin B in a 1:1 association (Labbé *et al.*, 1989a). In active MPF, the cdk1 subunit is unphosphorylated upon residues threonine 14 and tyrosine 15 (Norbury *et al.*, 1991; Borgne and Meijer, 1996) and phosphorylated upon residue threonine 161 (Lorca *et al.*, 1992; Fesquet *et al.*, 1993) or the equivalent residue threonine 167 in fission yeast (Gould *et al.*, 1991). Active MPF has this same form throughout the animal kingdom, however, the precise method of regulation and activation of MPF is dependent upon the species.

The level of cdk1 does not vary throughout the cell cycle (Simanis and Nurse, 1986; Lee and Nurse, 1987; Lee *et al.*, 1988; Arion and Meijer, 1989). In contrast, the amount of cyclin B fluctuates due to its periodic synthesis and degradation throughout the cell cycle. In many organisms cyclin B is synthesised prior to M-phase, and recruits the already present cdk1 subunits (Ookata *et al.*, 1992) to form "pre-MPF" (see section 1.5.1). In other organisms, the cdk1 subunits remain monomeric until the trigger for M-phase onset is received and cyclin B is then synthesised to form active MPF (see section 1.5.2).

In summary, prior to M-phase (at the G_2/M-phase border) cdk1 is present within the cell but maintained in one of two inactive forms; phosphorylated and bound to cyclin B or unphosphorylated and

monomeric. These alternative mechanisms for the regulation of MPF are discussed below.

1.5.1 Post-translational Activation of pre-MPF

"Pre-MPF" is the terminology given to the inactive latent form of MPF present at G_2/M-phase border of many eukaryotic organisms such as yeast (Nurse and Bissett, 1981) and the oocytes of *Xenopus* (Lohka *et al.*, 1988), starfish (Labbé *et al.*, 1989a, b) and mammals (Choi *et al.*, 1991). Cyclin B is synthesised during G_2 and recruits the already present cdk1 subunits to form pre-MPF (Solomon *et al.*, 1990; Hayles and Nurse, 1995). However, formation of this complex does not result in automatic activation of the kinase as binding to cyclin B induces the phosphorylation of specific inhibitory residues upon the cdk1 subunit (Solomon *et al.*, 1990; Parker *et al.*, 1991). The first inhibitory phosphorylation site is located at tyrosine 15 and found in all eukaryotic organisms so far studied (Gould and Nurse, 1989; Norbury *et al.*, 1991; Amon *et al.*, 1992). In higher eukaryotic cdk1, a second additional inhibitory residue at threonine 14 is phosphorylated (Krek and Nigg, 1991; Norbury *et al.*, 1991; Borgne and Meijer, 1996). Activation is achieved by the dephosphorylation of the(se) inhibitory residue(s) to convert the latent form of MPF (pre-MPF) to the fully functional form (see Fig.1.4).

Inhibition of MPF Activity

The dephosphorylation and phosphorylation of the cdk1 subunit is controlled by a series of protein kinases and protein phosphatases. The gene wee1 was first isolated in fission yeast (Nurse, 1975) and was so called as yeast strains which had a mutant wee1 gene were found to divide at a smaller size, and the Scottish term for small is "wee". Homologues to wee1 are also found in other organisms demonstrating the conservation of this gene throughout all eukaryotic cells (Featherstone and Russell, 1991; Igarashi

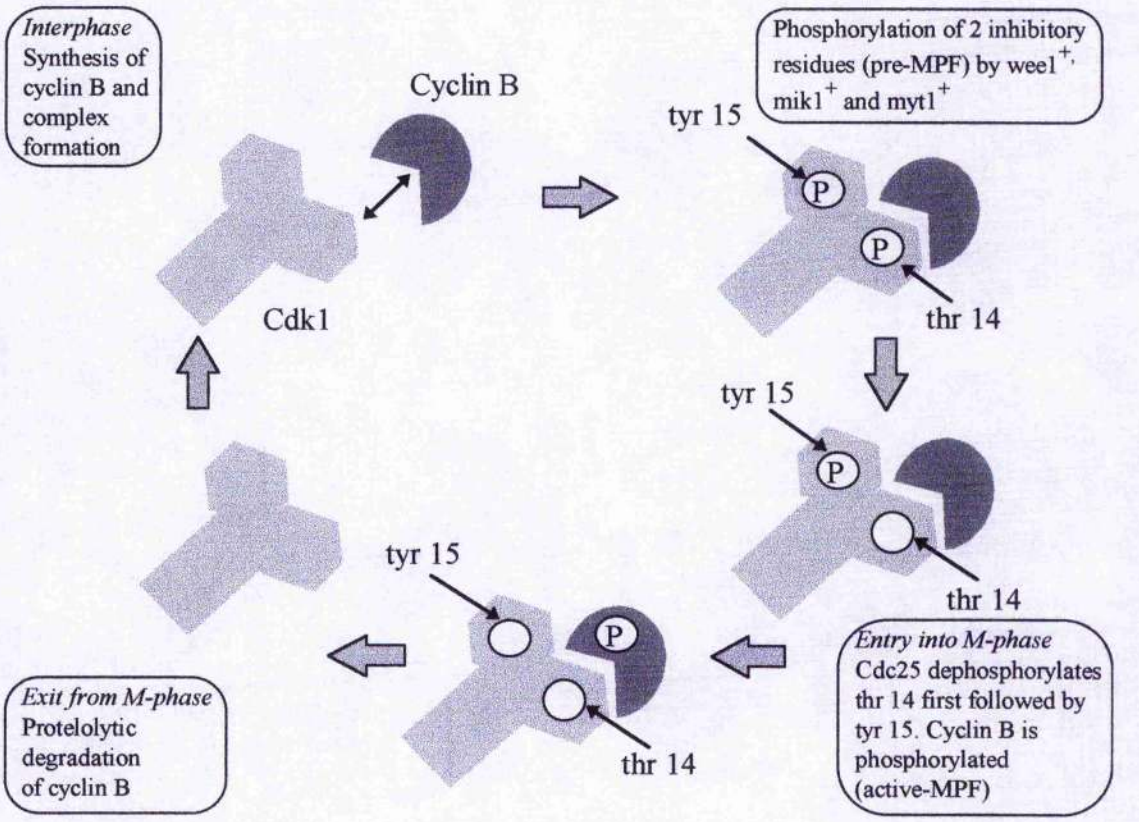


Figure 1.4: Post-translational Activation of MPF
(original figure)

et al., 1991; Booher *et al.*, 1993; McGowan and Russell, 1993; McGowan and Russell, 1995). The protein product of wee1 is a dual specificity kinase (Featherstone and Russell, 1991; Parker *et al.*, 1992) and hence has the potential to phosphorylate both inhibitory residues threonine 14 (Thr 14) and tyrosine 15 (Tyr 15) upon cdk1. However, *in vivo*, wee1 is responsible for the phosphorylation of Tyr 15 only (Parker *et al.*, 1992; McGowan and Russell, 1993; McGowan and Russell, 1995).

Other enzymes that have a role in the inhibitory phosphorylation of cdk1 are the protein products of myt1 (Mueller *et al.*, 1995; Liu *et al.*, 1997) and mik1 (Lundgren *et al.*, 1991; Lee *et al.*, 1994). As with wee1, the mik1 gene product catalyses the phosphorylation of cdk1 at Tyr 15 (Lee *et al.*, 1994). The myt1 protein product phosphorylates both Thr 14 and Tyr 15 (Mueller *et al.*, 1995). It is thought that the combined action of the protein products of the three genes wee1, mik1 and myt1 are responsible for the inhibitory phosphorylations on cdk1 at Thr 14 and Tyr 15 (Lundgren *et al.*, 1991; Parker *et al.*, 1992; Lee *et al.*, 1994).

Activation of MPF

The antagonist to these inhibitory kinases is the protein product of cdc25 which was originally discovered as the essential rate limiting inducer of mitosis in genetic yeast studies (Russell and Nurse, 1986). cdc25 is the activating phosphatase of cdk1 (Russell and Nurse, 1986; Moreno *et al.*, 1989; Sadhu *et al.*, 1990; Strausfeld *et al.*, 1991; Honda *et al.*, 1993). This protein is a dual specificity phosphatase that removes the inhibitory phosphate from both Tyr 15 and Thr 14 of pre-MPF complexes, creating the fully active enzyme (Strausfeld *et al.*, 1991; Jessus and Beach, 1992; Honda, et al., 1993). Another protein phosphatase isolated from fission yeast is the PTPase (Protein Tyrosine Phosphatase) pyp3 (Millar and Russell, 1992) that removes the phosphate group from Tyr 15 only (Borgne and Meijer, 1996). In

addition to the positive regulation of MPF by these phosphatases, the formation of functional cdk1/cyclin B is enhanced by negative regulation *via* the suppression of wee1 activity, during the G_2 to M-phase transition (McGowan and Russell, 1995).

The post-translational activation of MPF is augmented by autocatalytic amplification whereby the activation of MPF complexes induces the activation of others. The existence of self-autocatalytic amplification was first shown by Kishimoto and Kanatani (1976) using starfish oocytes. These experimenters micro-injected oocyte cytoplasm, containing MPF, into immature oocytes. These oocytes then matured and transfer of this cytoplasm was then micro-injected into other immature oocytes. This experiment demonstrated that serial transfer of maturing oocyte cytoplasm into prophase oocytes does not diminish MPF activity and hence must enhance its own activation (Kishimoto and Kanatani, 1976). More recent research shows that during the G_2 to M-phase transition, cdc25 exhibits increased phosphorylation which increases its phosphatase activity (Hoffmann *et al.*, 1993) and results indicate it is regulated *via* the kinase activity of cdk1/cyclin B complexes (Hoffmann *et al.*, 1993; Strausfeld *et al.*, 1994). These results suggest that the auto-amplification occurs due to the activation of a small number of MPF complexes, which in turn phosphorylate cdc25 that activates its phosphatase ability, active cdc25 then dephosphorylates cdk1 to create more active MPF complexes.

1.5.2 Activation by Cyclin B

In some animals, no pre-MPF complexes are formed because prior to M-phase the cdk1 subunits are monomeric. In this case, cyclin B is the activating factor. This mechanism of MPF regulation occurs in the oocytes of all teleost species investigated to date (Hirai *et al.*, 1992; Katsu *et al.*, 1993; Tanaka and Yamashita, 1995; Yamashita *et al.*, 1995; Haider and

Balamurugan, 1996; Kondo *et al.*, 1997), all amphibians investigated to date except *Xenopus laevis* (Tanaka and Yamashita, 1995; Ihara *et al.*, 1998; Sakamoto *et al.*, 1998) and the pig (Naito *et al.*, 1995). During the prophase I arrest in the oocytes of these organisms, cdk1 is present as a monomeric entity and initiation of meiosis is triggered by the synthesis of cyclin B which recruits the cdk1 subunits and forms active MPF.

1.5.3 Phosphorylation of Threonine 161

In contrast to Thr 14 and Tyr 15, the threonine residue at position 161 (167 in yeast) upon the cdk1 subunit must be phosphorylated for kinase activation to occur (Gould *et al.*, 1991; Fesquet *et al.*, 1993). Phosphorylation of this residue is required in both models of MPF activation ("post-translational dephosphorylation of cdk1" and "activation by cyclin B synthesis"). Threonine 161/167 is phosphorylated by CAK (cdk activating kinase) whose kinase subunit is encoded by the gene MO15 (Fesquet *et al.*, 1993). An investigation by Lorca *et al.* (1992) using *Xenopus* oocytes, indicates that phosphorylation of threonine 161/167 will only occur once a complex between cdk1 and cyclin B has formed. Furthermore, results show that the phosphorylation of this residue improves the stability of the complex (Gould *et al.*, 1991).

1.5.4 Role of Cyclin B

As the name "cyclin-dependent kinase" (cdk) suggests, association with cyclin is essential in order for cdk1 to obtain kinase activity. Cyclin B can therefore be considered as the fundamental regulator of cdk1 because initially it recruits the monomeric cdk1 subunits, association with cyclin B is a pre-requisite for onset of M-phase entry and its destruction allows the advancement into anaphase (see section 1.5.5). Further to this role, cyclin B has other properties. The work of Galaktionov and Beach (1991) shows that

cyclin B triggers the phosphatase activity of human cdc25, even in the absence of cdk1. This subunit, therefore, has another potential role in the regulation of cdk1, albeit indirectly, by activating cdc25. Furthermore, cyclin B is a phosphoprotein itself and during the G_2 to M-phase transition becomes phosphorylated (Pondaven *et al.*, 1990) although the significance of this is not yet known.

1.5.5 Exit from M-phase

Exit from M-phase is initiated by the destruction of cyclin B which inactivates the MPF complex and allows the cell to exit from metaphase (see Fig. 1.3) and proceed to anaphase (Standart *et al.*, 1987; Murray *et al.*, 1989). The threonine 161 residue upon the cdk1 subunit must also be dephosphorylated to inactive the kinase ability (Lorca *et al.*, 1992). Proteolysis of cyclin occurs first, freeing the cdk1 subunit, which allows the removal of phosphothreonine 161 (Lorca *et al.*, 1992) Evidence shows that dephosphorylation of threonine 161 is catalysed, at least in part, by type 1A phosphatase (Lorca *et al.*, 1992).

1.6 Reproductive Biology of the Three Species of Polychaete

This section discusses each of the polychaete species that were used for the research described in this thesis, with particular regard to the reproductive biology. Although oocyte maturation in the three species has been mentioned briefly in the introduction, a more extensive description is given for each species in the following section.

1.6.1 *Arenicola marina*

The lugworm, *Arenicola marina*, (L.) is a gonochornistic

(separate sexes) polychaete with external fertilization that inhabits a U-shaped burrow in the intertidal zone of sandy shores and estuaries (Wells, 1966; Williams *et al.*, 1997). This is an annual iteroparous species and maturity, in individuals from most natural populations, is reached during the second year (Newell, 1948; Duncan, 1960).

Oogenesis

Oocytes are released from the gonads at an early stage (early vitellogenic) and development occurs freely over a period of several months with the oocytes bathed in coelomic fluid (Rashan, 1980). The progress of the developing oocytes has been described in detail by Rashan (1980), a summary of which follows. The oogonia are enclosed initially within the ovary, where mitosis occurs, thereby increasing the numbers of primary oogonia. During this period the oogonia are observed with a large germinal vesicle but contain little cytoplasmic material. As development proceeds within the ovary, meiosis is initiated producing large numbers of primary oocytes. As the primary oocytes continue to develop, the plasma membrane becomes folded into numerous microvilli, cytoplasmic volume increases, and the yolk synthesis organelles (e.g. endoplasmic reticulum and golgi bodies) become abundant. Prior to release from the ovary, primary yolk body production has already begun with a small number of yolk granules dispersed throughout the cytoplasm. The oocytes are released into the coelomic cavity at approximately 17.5 - 25 μm in diameter. The cell cycle is arrested in the first prophase of meiosis and remains so until maturation and spawning. During the next 4 - 5 months the oocytes undergo a stage of active yolk production, vitellogenesis. During vitellogenesis, a number of cytoplasmic inclusions appear; two types of protein yolk granule, lipid droplets, and at a later stage the cortical granules form. In addition, the egg envelope forms around the primary oocytes during the beginning of the

coelomic phase.

Oogenesis appears to be a relatively unsynchronised process and the population of egg sizes within females is bimodal and shows great variability (Rashan and Howie, 1982). Synchronisation of egg sizes is gained in a very short period of time immediately prior to the spawning period, the stimulus for which is not known (Rashan and Howie, 1982).

Maturation

As mentioned above, oocyte development is arrested at meiotic prophase I until final maturation and spawning is initiated (Howie, 1961b; Howie, 1966; Watson and Bentley, 1997). Maturation is always followed by spawning, and the shedding of gametes occurs via the ciliated funnels of the nephridia (Howie, 1961a). Prior to maturation the unripe oocytes are rejected and Howie (1961a) suggested that the change in oocyte shape that accompanies maturation provides the signal for acceptance into the nephridia and hence to the exterior .

Maturation of *Arenicola marina* oocytes is controlled by at least two factors and results in the spawning of fertilizable oocytes (Watson and Bentley, 1997). The prostomial maturation hormone (PMH[2]) is released, which initiates the production of the second hormone, the coelomic maturation factor, CMF[2] (Watson and Bentley, 1997). Following injection of PMH into the female lugworm, CMF is produced after 1 to 2 hours (Watson and Bentley, 1998a). CMF acts directly upon the oocytes, as shown by *in vitro* studies, leading to entry into meiotic metaphase I (Watson and Bentley, 1997). As with 1-MeAde induction of starfish oocyte maturation, there is an

[2] The terms "hormone" and "factor" are used for PMH and CMF respectively because PMH is located in the prostomia and is released and acts at another part of the body (i.e. an hormone). The site of production/storage and site of activation of CMF has yet to be elucidated and is therefore called a "factor".

hormone dependent period during which the oocytes must be in contact with CMF for maturation to occur (Watson and Bentley, 1998a). Results show that CMF is a large molecule (> 30 kDa) that is heat-labile and inactivated by trypsin and hence, is probably a polypeptide (Watson and Bentley, 1997; Watson and Bentley, 1998a).

Watson and Bentley (1998b) followed the maturation process of *Arenicola marina* oocytes by immunofluorescence staining of the chromosomal material and the cytoskeleton coupled with CLSM and fluorescence microscopy. Entry into metaphase I is signified by chromosome condensation, GVBD and formation of the first meiotic spindle on which the paired chromosomes are aligned, at which stage meiosis is again halted until insemination (Watson and Bentley, 1998b). Furthermore, Rashan (1980) discovered that the cortical granules discharge their contents during the prophase to metaphase I transition, a perivitelline space is formed and the microvilli retract from the outer egg envelope.

Spermatogenesis and Maturation

As in some other polychaetes, development of the male gametes proceeds in the form of "sperm morulae" (Bentley and Pacey, 1989; Pacey and Bentley, 1992a). Each morula consists of bundles of immotile sperm, joined at the head to a common body of cytoplasm, the cytophore, where they remain until ripening and spawning is initiated (Bentley and Pacey, 1989; Pacey and Bentley, 1992a). As with the female gametes, unripe spermatozoa are rejected by the nephridia until maturation (Howie, 1961b). In addition, observations by Howie and McClenaghan (1965) and Olive (1972) indicate that spermatogenesis is under the control of an inhibitory feedback mechanism: removal of the gametes from the coelomic cavity results in a fresh burst of mitosis to increase the number of primary spermatocytes, but only in worms possessing intact brains.

Maturation of the spermatozoa occurs in response to the release of the male prostomial hormone and is followed by gamete shedding. The male prostomial hormone is called the sperm maturation factor (SMF) and has been putativley identified as the 20-carbon fatty acid 8, 11, 14-eicosatrienoic acid (Bentley *et al.*, 1990; Pacey and Bentley, 1992b). Release of SMF initiates the final ripening of the male gametes and the sperm morulae dissociate into free gametes (Pacey and Bentley, 1992a) which are released via the nephridia (Pacey and Bentley, 1992b). In addition to SMF, pH also has a role to play in the production of motile sperm. An investigation by Pacey *et al.* (1994) shows that following liberation from the cytophore, the motile apparatus of the sperm is switched on due to immersion in seawater with a higher pH. Furthermore, spawning in males is not a simply passive process but is accompanied by muscular contractions which result in successive convulsive movements that can last for over 1 hour (Pacey and Bentley, 1992).

Spawning Season

Spawning of most lugworm populations around the UK is typically an autumn or winter event (Duncan, 1960; De Wilde and Berghuis, 1979; Williams *et al.*, 1997), although springtime breeding populations have been reported (Howie, 1984). The spawning season of epidemic populations typically covers approximately one week during the months of October or November and is often associated with periods of spring tides (Duncan, 1960; Williams *et al.*, 1997). In contrast, the reproductive season of other populations, such as those found at Dunbar (Lothian, Scotland, UK) is more prolonged (pers. obs), lasting for approximately 2-3 weeks, and occurs later in the year (November/ December). In the case of epidemic spawning, the precise nature of the environmental factors imposing synchrony has yet to be confirmed, although links have been made with tidal cycles (Howie, 1984) and temperature (Howie, 1984; P. Cadman, pers. comm.). Worms collected

more than a month before the reproductive season and maintained in the laboratory (i.e. without tidal or daylength input) will spawn in the same week as their counterparts on the shore (Howie, 1963; Bentley and Pacey, 1992). It is believed, therefore, that synchronisation, at least in part, relies on an internal biological clock which is set by an external timing mechanism - the "zeitgeber" (Howie, 1984; Bentley and Pacey 1992).

Spawning is easily observed because the males produce distinctive sperm puddles upon the sand surface at low tide (Duncan, 1960; Bentley and Pacey, 1992; Williams *et al.*, 1997). In contrast, the females of this species spawn and retain their eggs within the burrow (Williams *et al.*, 1997). Transport of the spermatozoa is aided by the incoming tide (Williams *et al.*, 1997).

A preliminary study of the field fertilization success of *Arenicola marina* has been investigated in a population at St Andrews, Fife, Scotland (Williams *et al.*, 1997). The females were placed into artificial burrows (plastic U-shaped tubes) and transplanted to the field just prior to the spawning season. Fertilization success was found to vary between 0% and 90%, with values of approximately 50% characteristic of the females within the study (Williams *et al.*, 1997).

After fertilization, the embryos remain within their maternal burrow until reaching 2-3 segments and then migrate to the upper sediment layers, where they live in mucus tubes (Newell, 1949; Farke and Berghius, 1979). Following another growth period, the larvae migrate once more and produce their own U-shaped burrows often in "nursery" areas, before transferring to the adult population (Farke *et al.*, 1979).

1.6.2. *Arenicola defodiens*

The reproductive biology of the related species *Arenicola defodiens*

(Cadman and Nelson-Smith, 1993) has received less attention. Gamble and Ashworth (1898) investigated *Arenicola* populations and distinguished between *A. marina* and *A. defodiens* (although this name was not given at that time), however, for nearly a century, the two varieties were not differentiated in the scientific literature. Howie (1959) used the terms "laminarian" and "littoral" in his study of spawning in *A. marina*, however, he used this terminology "merely to indicate whether the worms were collected at a low level or high level on the shore" and hence did not consider them as separate species. Ironically, for many years anglers have always distinguished between the two species of bait using the terms "blow lug or red lug" (*A. marina*) and "black lug" (*A. defodiens*). A publication in 1990 (Cadman and Nelson-Smith, 1990) provided genetic evidence that the blow lug and black lug found on British shores were genetically distinct. Three years later, the black lug was described as a discrete species: "*A. defodiens*" (Cadman and Nelson-Smith, 1993). *A. defodiens* is morphologically similar to *A. marina* but has a thicker body wall, more elaborate gills, a black-coloured body and yellow tail as opposed to the dark red-brown body and greenish tail of *A. marina.* (Gamble and Ashworth, 1898; Cadman and Nelson-Smith, 1993). In addition, there are differences in habitat as *A. defodiens* is generally found in the low intertidal and subtidal regions, and *A. marina* are more predominant within the mid-intertidal zone (Gamble and Ashworth, 1898; Cadman, 1997). Furthermore, the burrow of *A. defodiens* is "J" shaped and generally descends deeper into the sediment (Cadman, 1997).

Meijer (Meijer and Durchon, 1977; Meijer, 1979a; Meijer, 1979b; Meijer, 1980) investigated various aspects of the morphology and biochemistry of oocyte maturation and fertilization in *Arenicola marina* [sic]. Since this time however, it has been shown that the animals studied by Meijer were *A. defodiens* (Watson *et al.*, 1998). Hence, this thesis will refer to Meijer's work as that completed upon *A. defodiens* not *A. defodiens.*

Oocyte Maturation

Oocyte maturation in *Arenicola defodiens* exhibits both similarities and differences compared to *A. marina. A. defodiens* oocytes arrest at prophase I until spawning is induced which results in the shedding of fertilizable metaphase I arrested oocytes (Meijer, 1979a; Meijer, 1979b; Watson *et al.*, 1998) through the nephridia (Meijer and Durchon, 1977). In addition to GVBD and formation of the metaphase spindle, the oocytes undergo other morphological changes. Meijer (1979a) used electron microscopy to examine such changes and these included a change in oocyte shape, microvilli retraction and cortical granule migration.

In contrast to *Arenicola marina*, evidence shows that oocyte maturation in *A. defodiens* is under the control of a single hormone. As with *A. marina*, a prostomial maturation hormone (PMH) is released which induces maturation and spawning (Watson *et al.*, 1998). However, incubation of immature oocytes with PMH *in vitro* results in the transition to metaphase I (Meijer and Durchon, 1977; Meijer, 1979b; Watson *et al.*, 1998). It is, therefore, apparent that PMH acts directly upon the oocytes *in vivo*, and no secondary coelomic derived hormone is required (Watson *et al.*, 1998).

Spermatogenesis

Spermatogenesis in *Arenicola defodiens* is similar to *A. marina*. Bundles of immature spermatozoa are joined together as a synctium, at a common cytophore (Meijer, 1979b). Injection of male prostomia *in vivo* and the incubation of morulae in prostomial extract *in vitro* induces dissociation from the cytophore to form fertilizable sperm (Meijer, 1979b). Male *A. defodiens* also respond to injection by 8,11,14-eicosatrienoic acid, and it is conceivable that this is also their true sperm maturation and spawning hormone (Watson *et al.*, 1998).

Spawning

Spawning of *Arenicola defodiens* populations around the British coast tends to occur later in the year than *A. marina*, typically during the months of December and January (Cadman, 1997).

1.6.3. *Nereis virens*

Nereis virens (Sars) is a gonochornistic polychaete that inhabits the intertidal muddy sands of marine and estuarine environments. It has several common names including the king ragworm, the sandworm and the clamworm. As with all nereid polychaetes, *N. virens* is semelparous, having a single spawning event which is followed by inevitable death.

Oogenesis

Life cycle studies have been undertaken upon *Nereis virens* from a Thames estuary population in the UK (Brafield and Chapman, 1967; Bass and Brafield, 1972), a Canadian population from the coast of New Brunswick (Snow and Rattenbury Marsden, 1974) and from the shore of the St Lawrence estuary (Desrosiers *et al.*, 1994).

The initial stages of oogenesis were recorded by Snow and Rattenbury Marsden (1974), a summary of which is given below. The oogonia are first observed as clusters of cells (gonadal clumps) found in the parenchymatous tissue or freely floating in the coelomic fluid. Growth continues within these gonadal clumps, until the oocytes reach 10-25 μm diameter. They then detach to become free floating coelomic oocytes. Within any given female, oocyte size is not uniform but shows wide variation (Brafield and Chapman, 1967; Snow and Rattenbury Marsden, 1974). During the late summer, sampling of females and investigation of their coelomic contents reveals that some individuals contained gonadal clumps and smaller oocytes of various

sizes (<90 μm), whereas other contained no gonadal clumps and oocytes of a larger size (>100 μm), (Snow and Rattenbury Marsden, 1974). Individuals with small oocytes can be found at any time throughout the year but the second subset (containing larger oocytes) is found during late July and August time only. During the late autumn and winter these larger oocytes undergo a period of rapid growth up to 240μm but no rapid growth period is observed in the subset of females containing smaller oocytes (Snow and Rattenbury Marsden, 1974). This suggests that individuals may carry a pool of small undeveloped oocytes for considerable periods of time and that individuals that are going to mature and breed in a given year enter vitellogenesis more or less synchronously in July. Only the subset of females with larger oocytes that have undergone this rapid growth phase will spawn the following year, and spawning occurs during the late spring/ summer (Snow and Rattenbury Marsden, 1974).

During oogenesis, the oocytes sequester yolk proteins into granules during vitellogenesis. An investigation by Fischer and Dhainaut (1985) used electron microscopy coupled with autoradiography to study yolk production. The study demonstrated that yolk precursors are derived from autosynthetic (protein synthesised by the oocyte itself) and heterosynthetic (protein incorporated from outside the oocyte) origins. Previous studies by Fischer (1979) and Fischer and Schmitz (1981) demonstrated the presence of a yolk protein-like substance in the coelomic fluids of *Nereis virens* females. This vitellogenin was found to be the precursor to the actual yolk protein, vitellin. Vitellogenin is produced by eleocytes, which are free floating cells present within the coelomic fluid, and taken up by the oocytes (Fischer and Rabien, 1986).

Spermatogenesis

Spermatogenesis takes less time from proliferation to maturation

than female gametes, and is completed in less than one year (Brafield and Chapman, 1967). As in females, male gametogenesis begins with gonadal clusters, but these differentiate into coelomic sperm plates during July and August (Snow and Rattenbury Marsden, 1974). As maturation continues, it becomes clear that these sperm plates are composed of only four spermatozoa (tetrads) joined together at the head regions (Brafield and Chapman, 1967). After a number of months the spermatozoa detach from their tetrad and become free but immotile in the coelomic cavity (Brafield and Chapman, 1967). By the beginning of May, all the sperm have dissociated and show high swimming activity when observed in seawater (Bass and Brafield, 1972).

Oocyte Maturation

Meiosis is arrested at the prophase I stage, with the germinal vesicle still intact (Bass and Brafield, 1972). Prior to spawning, *Nereis virens* oocytes undergo no final nuclear maturation (i.e. no meiotic reinitiation occurs), are expelled with intact germinal vesicles and the prophase block is only lifted once insemination occurs (Bass and Brafield, 1972). Furthermore, spawning is not a prerequisite for fertilizability, oocytes can be withdrawn from the coelomic cavity manually and fertilized *in vitro* .

Spawning

Oogenesis takes from between one and two years (Brafield and Chapman, 1967; Snow and Rattenbury Marsden, 1974). Brafield and Chapman (1967) stated that maturity probably occurred in the second or third year, yet the more extensive study of Snow and Rattenbury Marsden (1974) predicted that an age of 4 years old or over was more likely.

Prior to the spawning season, male *Nereis virens* begin to develop epitokous morphological changes. The parapodia increase in size,

natatory chaetae develop and degradation of the body wall occurs (Bass and Brafield, 1972). The males exhibit this partial morphogenesis in preparation for swarming: when ready to spawn the males leave their burrows and swim to the water surface where the gametes are released via pores within the purposefully formed anal rosette (Bass and Brafield, 1972; Desrosiers *et al.*, 1994). Once spent, the males die (Brafield and Chapman, 1967; Bass and Brafield, 1972; Snow and Rattenbury Marsden, 1974; Desrosiers *et al.*, 1994). Little change occurs in the morphology of the female, although the body wall becomes thinner and muscle wastage occurs in the more posterior regions where the oocytes are found (Brafield and Chapman, 1967). No effort is made to equip the female with pores for oocyte release and gamete shedding is achieved by the rupturing of the body wall (Bass and Brafield, 1972). Furthermore, in contrast to the males, the females of this species do not swarm but remain within their burrows and release oocytes onto the sediment surface (Bass and Brafield, 1972; Desrosiers *et al.*, 1994) before death inevitably ensues (Brafield and Chapman, 1967).

As mentioned above, spawning in *Nereis virens* is typically a late spring/ early summer event (Brafield and Chapman, 1967; Desrosiers *et al.*, 1994). Spawning is synchronised within the population (Brafield and Chapman, 1967; Bass and Brafield, 1972) and coincides with the appearance of a new moon and hence could be linked to lunar or tidal cycles (Brafield and Chapman, 1967). Furthermore, a premature rise in temperature, induced under laboratory conditions, can induce spawning, although the gametes produced are incapable of fertilization (Bass and Brafield, 1972). This indicates an important role for temperature in the control and induction of spawning.

Following fertilization and embryo formation, a larva is formed and is found upon the sediment surface (Snow and Rattenbury Marsden, 1974).

Under laboratory conditions, formation of the larva takes approximately 30 hours at 13°C (Bass and Brafield, 1972). Bass and Brafield (1972) reported on the presence of a brief planktonic trochophore larval stage, however, this was not found by Snow and Rattenbury Marsden (1974). These authors did not rule out completely a larval stage but stated the likelihood that larval development was totally benthonic. However, in the much more recent investigation, Desrosiers *et al.* (1994) also claimed that larval undergo both a benthonic and pelagic development which is followed by settlement in the upper intertidal zone and after 2 years, the juveniles migrate down shore into the adult population.

1.7 Invertebrate Systems - a Worthwhile Model?

In a world where science is driven towards the applied approach that will contribute to wealth creation and improving the quality of life, the relevance of invertebrate research may be questioned. Nevertheless, research such as that described in this thesis, which uses an invertebrate model, is vital and not purely for the expansion of knowledge. The basic and fundamental principles discovered during invertebrate systems apply to all organisms, including mammals (Yamashita *et al.*, 1999). The use of marine invertebrates is particularly advantageous in gamete research because spawning is often synchronised across the population. Hence within a specific population of a particular species, there are many individuals at the same stage in development available for study. The animals require simple maintenance, and fecund individuals produce large quantities of gametes, which are easily obtained and cultured (i.e. in seawater). In addition, the biological systems within invertebrates are less complex. Invertebrates are therefore an ideal model for the study of systems such as hormone regulation, gametogenesis, gamete maturation, spawning and

fertilization. For example, the sea urchin has been an extremely important model to study aspects of fertilization biology, especially the role of calcium as the intracellular signal (Whitaker and Swann, 1993). Starfish have been of central importance to the studies of oocyte maturation and hormone regulation (Meijer and Mordret, 1994).

Oocytes and eggs of marine invertebrates (mostly starfish to date) also provide excellent cellular models to study cell division and its intracellular regulation. These cells have contributed greatly to our understanding of this essential biological process, shared by all living organism, from yeast to humans (see section 1.4.1).

Tumour development in humans is associated with the deregulation of the cell cycle which is associated with a malfunctioning cdk regulatory network (Motokura and Arnold, 1993; Peters, 1994). The cdk1/cyclin B kinase is now widely used by major pharmaceutical companies around the world as a molecular target in their screening efforts for the discovery of novel anti-tumour agents (Meijer, 1996). Starfish oocytes are the best known source for the purification of this major cell cycle regulator.

Do we need another marine invertebrate model for cell cycle regulation? The study of new cellular models will provide innovative approaches to cell cycle studies and may lead to new applications in the cancer field. The next section describes why polychaete oocytes are such an apt model to investigate these processes.

1.8 Advantages of Working with Polychaete Oocytes

The use of polychaete oocytes are excellent models for investigation

into oocyte maturation and the control of meiotic reinitiation. During the spawning season, each female polychaete provides a large, homogeneous and synchronous population of oocytes. The oocytes are easily accessible (freely floating as opposed to being within an ovary) and solitary (not associated with follicle cells). All the oocytes are arrested at G_2/prophase I border and meiotic reinitiation can be induced at will by the application of CMF, in the case of the *Arenicola marina*, PMH, in the case of the *A. defodiens* and sperm with respect to *Nereis virens*. Furthermore, meiotic maturation results in GVBD, providing a large morphological change which can be easily detected. The oocytes require no special culturing media or equipment and can be incubated in seawater.

1.9 Aims and Objectives

Over the last 10 years, a considerable amount of research has been dedicated towards the hormonal mechanisms that control gamete maturation in the lugworm, *Arenicola marina* (Bentley, 1985; Pacey and Bentley, 1992a; Pacey and Bentley, 1992b; Watson and Bentley, 1998a; Watson and Bentley, 1998b; Watson *et al.*, 1998). With respect to *A. defodiens*, the research on hormonal gamete maturation was investigated by Meijer (Meijer and Durchon, 1977; Meijer, 1979a; Meijer 1979b; Meijer, 1980) and has been continued by Watson *et al.* (1998). Research upon *Nereis virens* has focused primarily towards the control of oogenesis and vitellogenesis (Fischer, 1979; Fischer and Schmitz, 1981; Fischer and Rabien, 1986; Fischer *et al.*, 1991). In each of the three species, little work has been directed towards the intracellular regulation of meiotic reinitiation during oocyte maturation. Furthermore, compared to other organisms, such as echinoderms, molluscs, amphibians, fish and mammals, the knowledge of MPF in the polychaetes is scarce and has been completed upon only one species, *Chaetopterus*

pergamentaceus (Eckberg *et al.*, 1996; Eckberg, 1997). The research described in this thesis focuses on the intracellular regulation of meiosis in polychaete oocytes during oocyte maturation. Investigations are also directed towards the extracellular hormonal control of oocyte maturation in *A. marina*.

The aims of this thesis are:

- To characterise the ultrastructural changes during meiotic maturation using electron microscopy in the oocytes of *Arenicola marina* and *Nereis virens*
- To further the studies of hormone regulation in *Arenicola marina* oocyte maturation
- To investigate the intracellular effectors that are involved in the signal transduction pathway from the hormone to MPF activation in the oocytes of *Arenicola marina* and *A. defodiens*
- To investigate the regulation and activation of MPF during the meiotic reinitiation in the oocytes of *Arenicola marina* and *Nereis virens*
- To investigate the inhibition of meiotic reinitiation in *Arenicola marina* oocytes, by chemical agents.

CHAPTER 2

ULTRASTRUCTURE OF MEIOTIC MATURATION IN THE OOCYTES OF *NEREIS VIRENS*

2.1 INTRODUCTION

The oocytes of *Nereis virens* are spawned at prophase of meiosis I, also known as the germinal vesicle stage. Meiosis is resumed by insemination and the ultrastructural aspects of this event have been documented, using transmission electron microscopy, TEM (Bass and Brafield, 1972). The pictorial description provided by these authors is, however, very limited and hence a more extensive study was clearly required. As stated by Bass and Brafield (1972), their study was restricted by poor tissue preservation due to the extracellular jelly coat that forms following fertilization. Other authors have also reported difficulty in the fixation of marine invertebrate oocytes (Eisenman and Alfert, 1982).

The timing of germinal vesicle breakdown (GVBD) in the oocytes of *Nereis virens* has yet to be determined. The oocytes are filled with green-coloured yolk that obscures the germinal vesicle and prevents observation of when GVBD occurs. Watson (1996) attempted to alleviate this problem by fixing the oocytes and staining with fluorescent dyes which bind to DNA and the microtubules. Nonetheless, preservation of the oocytes for fluorescence microscopy was unsuccessful due to inadequate penetration of the fixatives (Watson, 1996). The ability to score oocytes for GVBD was, however, required for studies described later (see Chapter 6).

There is an extensive range of fixation procedures available for microscopy studies. These include a variety of primary fixatives, buffers, additives, embedding media, and times and temperatures for fixation. In tissues which are difficult to fix, a number of fixation protocols can be tried and tested. In addition, the jelly layer can be removed from marine invertebrate oocytes (Speksnijder and Dohmen, 1983) which should enhance fixative penetration and consequently improve morphological preservation.

The removal of the jelly layer from fertilized *Nereis virens* oocytes has been attempted but was unsuccessful (Watson, 1996). Research shows that the use of microwaves during fixation, however, can increase chemical infiltration, decrease processing time and provide tissue preservation of the same quality or better than that of conventional chemical fixation (Leong *et al.*, 1985; Login and Dvorak, 1988; Jones and ap Gwynn, 1991; Giberson and Demaree, 1995). Fixation by microwaves requires certain factors to be taken into account and standardised if results are to be reproducible (Leong *et al.*, 1985; Login and Dvorak, 1988; Giberson and Demaree, 1995). The most important criterion is the final temperature of the fixative following exposure to the microwaves. Although given specimens will have an optimum temperature, a final post-irradiation fixative temperature of 50°C generally yields good results in a wide variety of tissues (Leong *et al.*, 1985; Login and Dvorak, 1988)

The aim of the studies in this chapter is initially to determine the best TEM fixation procedure for the morphological preservation of fertilized *Nereis virens* gametes. The second aim is to describe the ultrastructural changes during the early stages of fertilization and then finally, to establish a fixation method for fertilized oocytes to permit a fluorescence microscopy assay for scoring GVBD.

2.2 Materials and Methods

2.2.1 Collection and Maintenance of Animals

Gravid specimens of *Nereis virens* were obtained from a polychaete aquaculture farm, Seabait Ltd (Ashington, Newcastle upon Tyne). The worms were kept in individual pots (volume = 300ml) containing some gravel or stones, filled with 250 ml of filtered seawater (FSW, Gelman

sciences culture capsule filter, 0.5 μm) and maintained at a temperature of either 4 or 10 °C.

2.2.2 Gamete Collection

Assessment of Gamete Viability

Before fertilization and fixation, the gametes from each individual were assessed for their viability. Small samples of sperm were withdrawn from each male, diluted in twice filtered seawater (TFSW) and observed by light microscopy. Males were used as gamete donors if a substantial proportion (approximately > 60 %) of their sperm were highly motile. Fertilizability of the oocytes cannot be determined by observation alone, so small samples were taken from each female, mixed with sperm and left at 10°C for 15 minutes. The production of a jelly coat was taken as an indication of successful fertilization.

Fertilization of the Gametes

Oocytes were withdrawn from the coelomic cavity of each female ragworm (containing "fertilizable" oocytes) using a 1 ml disposable hypodermic syringe fitted with a 25g needle. Two hundred and fifty microlitres of the prophase I oocytes were aliquoted into 50 μl samples, washed in TFSW and fixed for TEM using 1 of the 4 methods described below (section 2.2.3). The remaining oocytes were placed in 1 litre of TFSW and placed at an ambient temperature of 10°C. Sperm was then removed from males using a 1 ml disposable hypodermic syringe (fitted with a 25g needle) and added to the 1 litre of seawater (final concentration 10^5 sperm.ml^{-1}) containing the oocytes and gently stirred. After 5 minutes the excess seawater was decanted (to remove surplus sperm) and replaced with fresh TFSW. Fertilized oocyte samples (250 - 500 μl) were withdrawn at 10

minute intervals (up to 100 minutes), washed in TFSW and fixed as described in section 2.2.3.

2.2.3 Fixation for Transmission Electron Microscopy

Primary Fixation

The gametes were fixed for TEM using 1 of 4 fixation protocols. The primary fixation procedure for each protocol is given in Table 2.1 and full details are given below for microwave assisted fixation (protocol 2). Details of post-fixation, dehydration and embedding for all 4 protocols are also described below.

Microwave Assisted Fixation

The term "microwave assisted fixation" (MAF) is used as this protocol combines the use of chemical fixatives with microwave irradiation. A conventional microwave oven was used with a 750W output and a digital control panel. The rotating plate inside the microwave was removed and replaced with a square Perspex plate that was raised by 2 cm on Perspex legs. A plastic beaker containing 400 ml of cold tap water was placed in the left-hand corner of the oven cavity to act as a "heat load".

To calibrate the microwave oven, a 10 ml glass vial containing 5 ml of fixative was placed in the centre of the oven cavity. The microwave was programmed to 50% power output and the fixative was irradiated for a pre-determined time (between 5 and 60 seconds). The vial was then removed and the temperature of the fixative was taken promptly using a mercury in glass thermometer. After each irradiation, a clean vial containing fresh fixative solution was used and the water used for the heat load was replaced. A range of times were tested and it was found that between 10 and 12 seconds was required for the temperature to reach 50 - 55°C.

Fixation Protocol	Fixation Type	Primary Fixative	Time and Temperature of Primary Fixation
1	Chemical fixation	2.5 % glutaraldehyde*, 2.5% formaldehyde**, 0.3M NaCl, 1% sucrose in seawater	Incubated for 1 hour at room temperature or 24 hours at 4°C
2	Microwave assisted fixation (MAF)	2.5% glutaraldehyde*, 2.5% formaldehyde**, 0.3M NaCl, 1% sucrose in TFSW	Placed into a 750W microwave oven and irradiated for 10-12 seconds at 50% power output (final irradiation T□ = 50-55°C)
3	Chemical fixation (from Coggeshall, 1972)	2.5% glutaraldehyde*, 2.5% formaldehyde**, 0.1% $CaCl_2$ 25% sucrose in 0.1M sodium cacodylate	Incubated for 1 hour at room temperature
4	Chemical fixation with pre-primary fixation with osmium (from Eisenmann and Alfert, 1982)	*Pre-primary fixative*: 2.5% glutaraldehyde*, 2.5% formaldehyde**, 0.1M NaCl, 0.02M $CaCl_2$ 1% sucrose 0.05% osmium tetroxide* in 0.1M sodium cacodylate *Primary fixative*: 2.5% glutaraldehyde*, 2.5% formaldehyde**, 0.1M NaCl, 0.02M $CaCl_2$, 1% sucrose in 0.1M sodium cacodylate	Incubated in pre-primary fixative for 10 minutes followed by 1 hour in primary fixative at room temperature

* EM grade, Agar scientific Ltd
** Made from paraformaldehyde, Agar scientific Ltd

Table 2.1: Primary fixatives used for each of the fixation protocols

Once the microwave was calibrated, the gametes of *Nereis virens* were fixed. A sample of gametes was placed into 5 ml of fresh fixative solution (see protocol 2 in Table 2.1 for ingredients) in a 10 ml glass vial and microwaved for a minimum of 10, and up to 12 seconds. If the post-irradiation temperature was within the range 50 – 55°C, the gametes were removed and quickly immersed into cold buffer rinse solution. However, if the fixative temperature was outside this range, the specimens were

discarded and the process was repeated with fresh tissue.

Post-fixation, and Dehydration - All Protocols

Following primary fixation, the specimens were rinsed in the buffer used for the fixative vehicle (i.e. the same solution used for the primary fixative but without the addition of aldehyde chemicals). This was followed by post-fixation of 1% osmium tetroxide (Agar Scientific Ltd, EM grade) in the same buffer and then rinsed once more for 15 minutes. Dehydration was carried out in ethanol series: 10 minutes in each 50%, 70%, 96% and 3 changes of 100% ethanol. Half of the specimens were embedded in LR White medium grade acrylic resin (Sigma) and the remaining samples were embedded in epoxy resin (TAAB laboratories Equipment).

LR White - All Protocols

The 100% ethanol was removed from each of the samples and replaced with LR white resin and left at 4°C for 12 to 18 hours. Following one change of resin, the samples were placed onto a rotator for approximately 4 hours and then embedded in fresh resin using gelatine capsules. The blocks were polymerised at 60□C for 24 hours.

Epoxy Resin - All Protocols

The remaining specimens were prepared for epoxy resin embedding. The absolute ethanol in each of the sample vials was replaced with an ethanol : propylene oxide mixture (1:1) for 15 minutes and then replaced with 100 % propylene oxide (2 x 15 minutes). Half of the propylene oxide was then removed from each sample and replaced with freshly mixed epoxy resin mixture. The samples were left for 12 to 18 hours in this mixture of propylene oxide and resin under constant rotation. After this period and following two changes of fresh resin over 12 hours, the samples were

embedded and the polymerised at 60°C for 48 hours.

Light Microscopy

Large (up to 2 mm square) 1µm semi-thin sections were cut from the embedded sample blocks and placed onto a drop of distilled water on a microscope slide. The slide was placed on a hotplate until all the water had evaporated and was replaced with a drop of toludine blue solution (0.5% toludine blue, 0.25% borax in distilled water). The slide was replaced on the hotplate for up to 1 minute before removing and rinsing with distilled water. The sections on the slide were then examined by light microscopy.

Transmission Electron Microscopy

Silver or silver/gold coloured sections were cut from each of the blocks and mounted on to 200 or 400 square mesh copper grids (previously coated in collodion film). The sections were stained using lead citrate solution followed by uranyl acetate solution and examined using a Phillips 301 TEM.

2.2.4 Microwave Fixation for Fluorescence Microscopy

The oocytes were placed in 5 ml of fluorescence microscopy fixative (2% formaldehyde in 20mM PIPES, pH 6.8, 5mM EGTA, 0.5mM $MgSO_4$ and 0.1% Triton X-100) and fixed by MAF as described for TEM (section 2.2.3). The oocytes were then removed and placed into cold fluorescence microscopy fixative and placed at 4°C for 25 minutes. The oocytes were then placed into the fluorescence microscopy rinse (same ingredients as given for the fixative without the inclusion of formaldehyde) and used immediately or stored at 4°C. For DNA labelling, the oocytes were washed in TFSW and 90 µl aliquots of the oocyte suspension were placed in a 96-well plate. Ten microlitres of Hoechst 33258 (10 $\mu g.ml^{-1}$ of TFSW) was added to each oocyte

sample. The oocytes were mounted on to slides with coverslips and observed by fluorescence microscopy (using a NPL FLUOTAR objectives with a Leitz Dialux 20 microscope with a HBO 50 W/AC mercury short arc lamp with filter block A, exciting at 340-380 nm and suppressing at 430 nm).

2.3 RESULTS

2.3.1 Morphological Quality

Fixation of fertilized oocytes by MAF for fluorescence microscopy was, however, unsuccessful as the morphological preservation was poor and the DNA staining was unsuccessful. A method to assess GVBD in a large number of oocytes was, however, developed. Large (up to 2 mm square) semi-thin (1μm) sections were cut from the LR white embedded samples (prepared for TEM) and stained and observed by light microscopy (Figs 2.1 and 2.2). Due to the size of the sections, up to approximately 20 - 25 oocytes could be examined at one time and assessed for the presence of a germinal vesicle.

Figures 2.3 to 2.7 and figures 2.9 to 2.20 depict micrographs, all fixed using MAF. As shown in figure 2.8, conventional chemical fixation (fixation protocols 1,3 and 4) results in substandard ultrastructural preservation. The oocytes are poorly fixed and have very low contrast (Fig. 2.8). As the jelly coating thickens, penetration of the fixatives becomes highly inadequate rendering the tissues and the organelles unrecognisable. MAF is the most successful fixation protocol for TEM and provides superior tissue preservation before and after jelly coat production (cf. Figs 2.4 and 2.8).

2.3.2 Ultrastructure of the Prophase I Oocyte

Meiosis is arrested at the prophase I stage, with an intact

germinal vesicle, until insemination occurs (Fig. 2.19). The oocytes (which range between 170 - 180 μm just prior to spawning) are fringed with a microvillus border (Figs 2.3 and 2.10). The unbranched microvilli arise from the oocyte plasma membrane (Fig. 2.10) and penetrate a vitelline layer that consists of three regions (Fig. 2.4). The inner tubular layer is moderately electron dense and permeated by canals (Fig. 2.4). Some of these canals house the microvilli but many are vacant (Figs 2.4, 2.9 and 2.10). This inner canal layer is edged with a thin electron-dense layer, upon which lies a thin granular outer coating (Figs 2.4 and 2.9) called the fibrous layer by Bass and Brafield (1972). At the oocyte periphery, many cortical granules are found (10 - 15 deep) containing whorls of fibrous material (Fig. 2.3). In addition, secondary cortical organelles are found at regular intervals along the oocyte plasma membrane (Fig. 2.9). The cytoplasm is filled with numerous large protein yolk platelets and lipid droplets (Fig. 2.3) plus some golgi bodies and ribosomes (Fig. 2.16). The germinal vesicle is rounded, contains a nucleolus, and is surrounded by numerous small mitochondria (Fig 2.18 and 2.19)

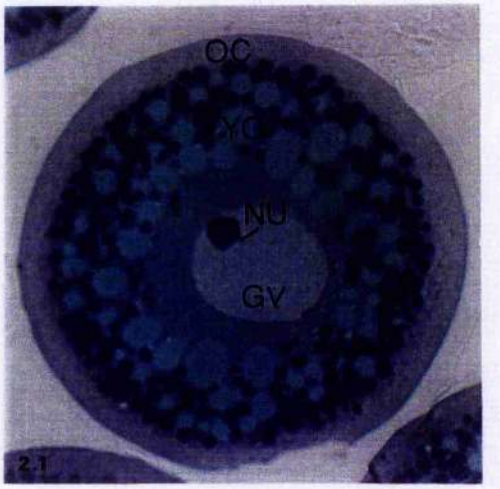
2.3.3 Early Events of Fertilization

Fertilization is readily apparent by the mass exocytosis of the cortical granules (Figs 2.4, 2.6 and 2.11). Only cortical granules at the oocyte plasma membrane undergo exocytosis (Figs 2.4, 2.6 and 2.11). In some cases, the cortical granules enter the perivitelline space, still intact and bound by membrane, and then empty their contents (Figs 2.4 and 2.11). In other cases, the cortical granules first fuse with the oocyte plasma membrane and then the material exudes out into the perivitelline space (Figs 2.4, 2.11 and 2.15). Following exocytosis, the cortical granules contents are observed in the perivitelline space, alongside the remains of the membranes (Figs 2.5, 2.6 and 2.11), and then expelled outside the vitelline layer to form the jelly coating (Figs 2.4 and 2.11). As post-fertilization time increases, cortical granule

exocytosis continues creating large cavities in the oocyte cortex, the perivitelline space becomes wider and the jelly layer thickens (Figs 2.6 and 2.12) and excess sperm become embedded within this gelatinous coating (Fig. 2.17). Although there is no major morphological change or elevation observed in the vitelline layer following insemination (Figs 2.5, 2.13 and 2.14) this coating is now referred to as the fertilization envelope. The only change observed between the fertilization envelope and the vitelline layer from which it was formed is that the outer granular coating begins to disintegrate after 10 minutes (Fig. 2.5) and is completely removed by 60 minutes post-fertilization (Fig. 2.6). Following insemination, the microvilli remain embedded within the fertilization envelope and are not withdrawn or retracted (Fig. 2.14). In Figs 2.6 and 2.13 the microvillus appears to be severed, but this is more likely to be due to sectioning, whereby the mid-piece of this microvillus was not within this slice. As depicted in Fig. 2.14, the microvilli remain intact but become elongated, extending from the oocyte plasma membrane to the fertilization envelope. Another organelle is present amongst the cortical granules (Fig. 2.12) and also remains at the oocyte cortex following completion of exocytosis (Fig. 2.14). This organelle is electron dense and contains thin regions of electron lucent material (Fig. 2.14). Between 80 and 90 minutes post fertilization, the germinal vesicle membrane breaks down (Fig. 2.20). After 100 minutes, exocytosis is complete, the perivitelline space decreases and the oocyte plasma membrane regains a smooth appearance (Fig. 2.7).

Figure 2.1: Unfertilized oocyte fixed by MAF and stained using toludine blue. The oocyte cortex (OC) contains many cortical granules, beneath which lies many lipid and protein yolk granules (YG). The germinal vesicle (GV) is large and contains a nucleolus (NU). Oocyte diameter = 180 μm.

Figure 2.2: Fertilized oocyte fixed by MAF (60 minutes post-fertilization) and stained using toludine blue. The oocyte cortex has decreased in size due to cortical granule exocytosis. Oocyte diameter = 180 μm.



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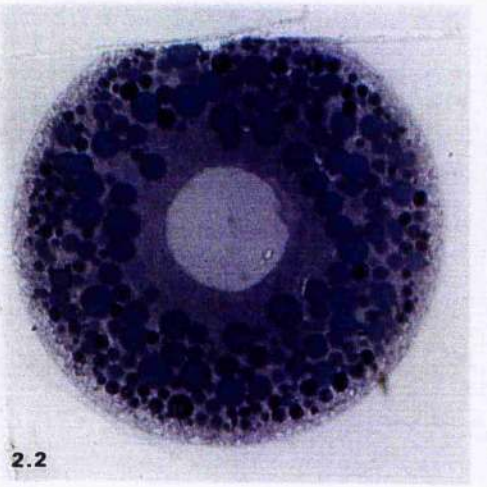

2.2

Figure 2.3: Unfertilized oocyte fixed by MAF. The outer region of the oocyte contains a dense population of cortical granules (CG) beneath which lie the protein yolk granules (YG). vitelline layer (VL); microvilli (MV). Scale bar = 2 μm.

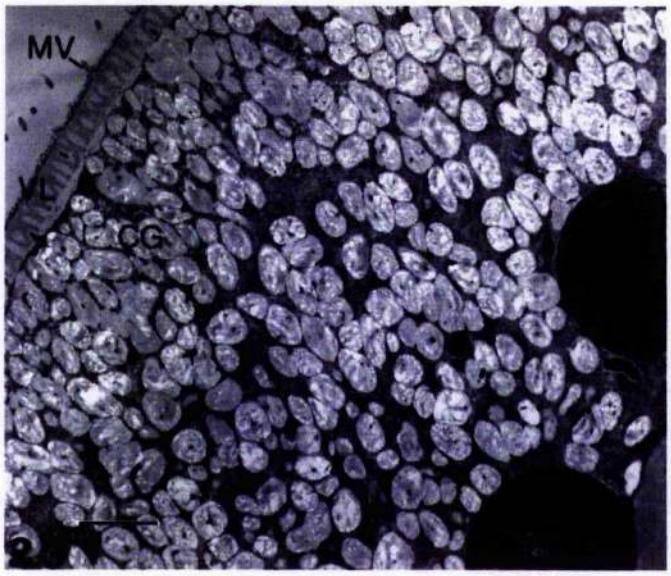
Figure 2.4: 10 minutes post-fertilization fixed by MAF. The cortical granules contain whorls of fibrous material which are released by exocytosis into the perivitelline space (PVS) and then through the fertilization envelope to form the jelly layer (JL). outer granular coating (GC); inner tubular layer (TL); thin electron dense mid-layer (ML). Scale bar = 0.5 μm.

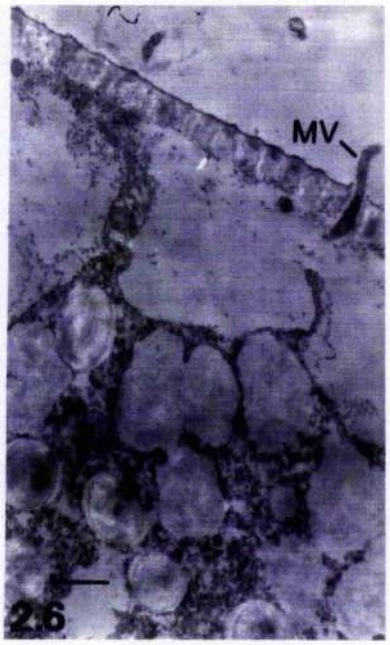
Figure 2.5: 30 minutes post-fertilization fixed by MAF. The cortical granules have broken down releasing the jelly precursor (JP) alongside the membrane remains (arrows) within the perivitelline space. Note the outer granular coating (GC) is disintegrating. oocyte plasma membrane (OPM). Scale bar = 0.5 μm.

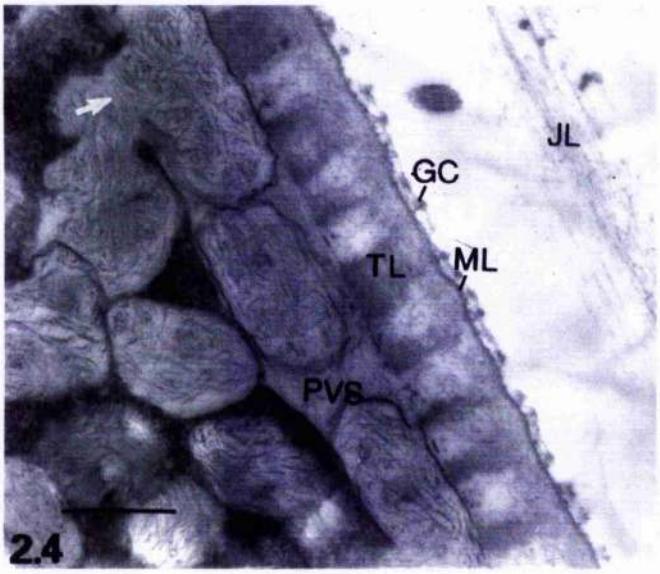
Figure 2.6: 60 minutes post-fertilization fixed by MAF. Cortical granule exocytosis continues, creating large cavities in the oocyte cortex. The outer granule layer has been completely removed from the oocyte surface. microvilli (MV). Scale bar = 0.5 μm.

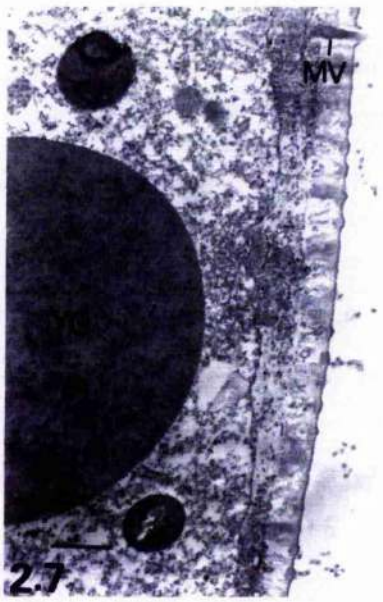
Figure 2.7: 100 minutes post-fertilization fixed by MAF. Cortical granule exocytosis is complete, the perivitelline space has decreased and the oocyte plasma membrane resumes a smooth appearance. microvilli (MV), yolk protein granule (YG). Scale bar = 0.5 μm.

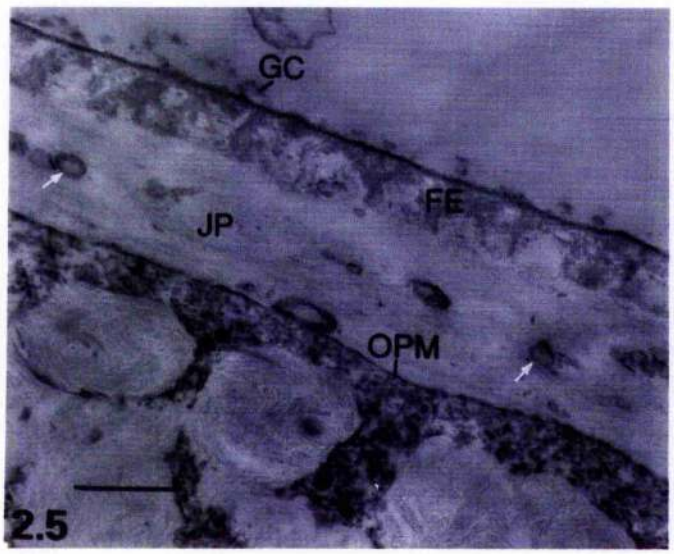
Figure 2.8: 60 minutes post-fertilization fixed using conventional chemical fixation. Morphological preservation of the fertilized oocyte is very poor. cortical granule (CG); fertilization envelope (FE). Scale bar = 0.5 μm.


MV
VL
CG


MV
2.6


JL
GC
TL
ML
PVS
2.4


MV
2.7


GC
FE
JP
OPM
2.5

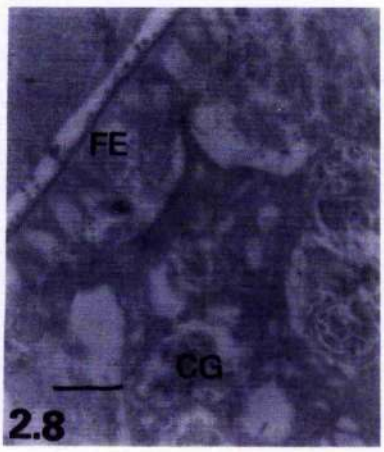

FE
CG
2.8

Figure 2.9: Unfertilized oocyte fixed by MAF. The oocyte cortex contains another cortical organelle (CO) situated at regular intervals next to the plasma membrane. Scale bar = 0.5 μm.

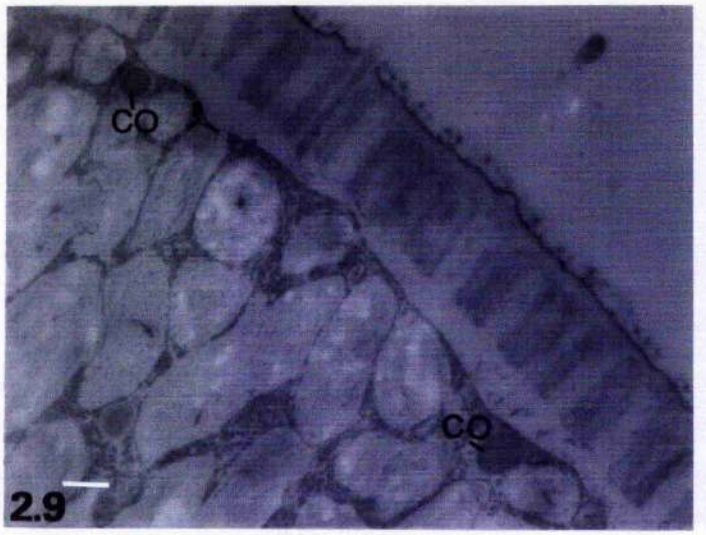
Figure 2.10: Unfertilized oocyte fixed by MAF. The microvilli (MV) arise from the oocyte plasma membrane and extend through the vitelline layer (VL). Scale bar = 0.5 μm.

Figure 2.11: 10 minutes post-fertilization fixed by MAF. Cortical granule exocytosis occurs in two ways. Either, the whole cortical granule is freed into the perivitelline space (large arrow) and releases the jelly precursor (JP), or the contents are expelled from the granule, whilst still in the oocyte cortex (small arrow). jelly layer (JL). Scale bar = 0.5 μm.

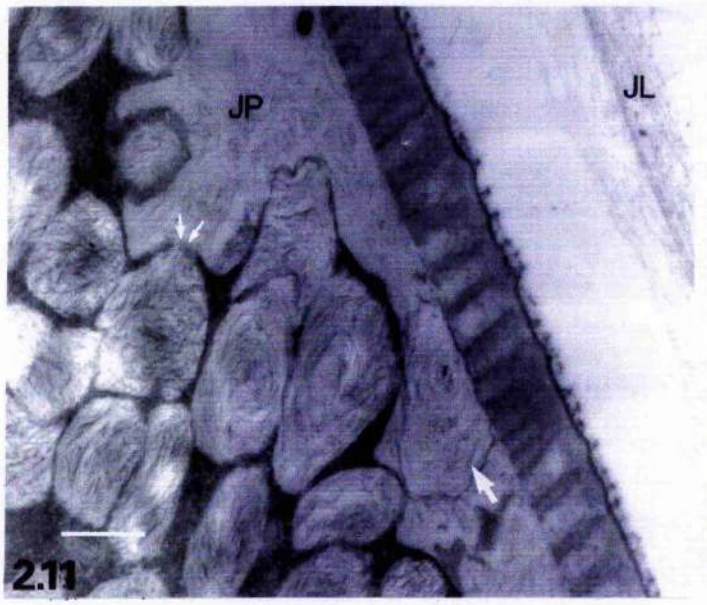
Figure 2.12: 80 minutes post-fertilization fixed by MAF. Note the almond-shaped organelle within the oocyte cortex (arrow). Scale bar = 0.5 μm.

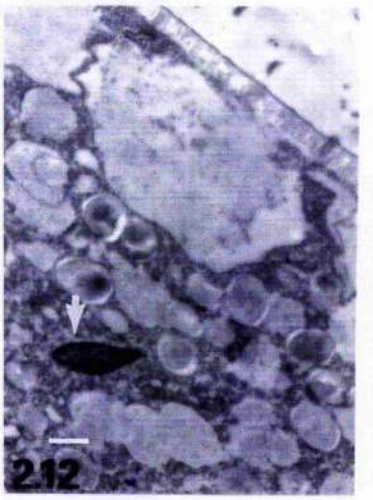
Figure 2.13: 60 minutes post-fertilization fixed by MAF (higher magnification of Figure 2.6). fertilization envelope (FE); perivitelline space (PVS). Scale bar = 0.5 μm.

Figure 2.14: 100 minutes post-fertilization fixed by MAF. The microvilli (MV) is elongated and extends from the oocyte plasma membrane (OPM) across the perivitelline space to the fertilization envelope (FE). Note the organelles (also observed in Fig. 2.12) at the oocyte plasma membrane. Scale bar = 0.5 μm.


CO
CO
2.9


MV
VL
2.10


JP
JL
2.11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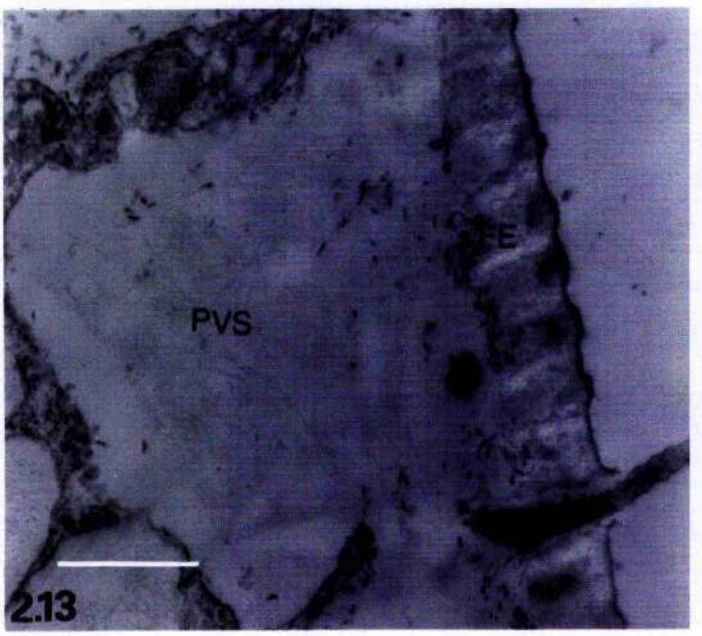
PVS
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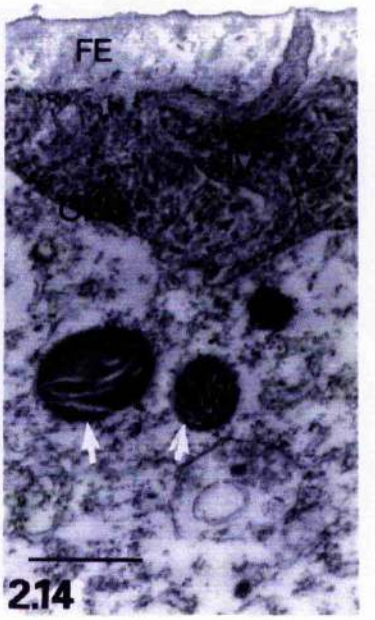

FE
2.14

Figure 2.15: 10 minutes post-fertilization fixed by MAF. Tangential section of the oocyte cortex showing the canals in the tubular layer, with the microvilli (MV) housed within but the majority are vacant (arrows). Scale bar = 0.5 μm.

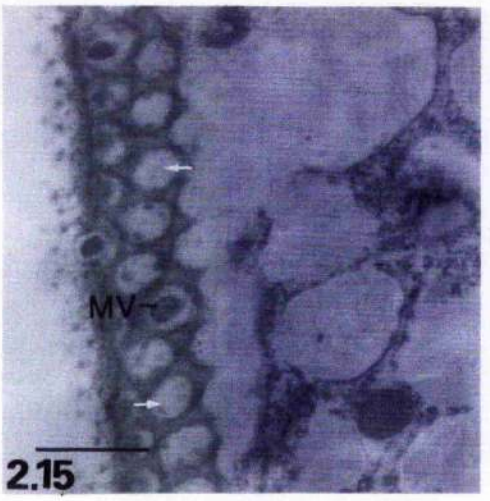
Figure 2.16: 40 minutes post-fertilization fixed by MAF. Showing a golgi body (GB) within the oocyte cytoplasm. Scale bar = 0.5 μm.

Figure 2.17: 20 minutes post-fertilization fixed by MAF. A sperm (SP) is trapped and embedded within the jelly layer (JL). Scale bar = 0.5 μm.

Figure 2.18: 60 minutes post-fertilization fixed by MAF. The area surrounding the germinal vesicle is surrounded by mitochondria (MI) and ribosomal (R) particles. Scale bar = 0.5 μm.

Figure 2.19: Unfertilized oocyte fixed by MAF. The germinal vesicle (GV) is rounded and contains a nucleolus (NU), and is surrounded by numerous mitochondria (MI). Scale bar = 2 μm.

Figure 2.20: 90 minutes post-fertilization fixed by MAF. The germinal vesicle membrane has broken down. chromatin (CH); mitochondria (MI). Scale bar = 2 μm.



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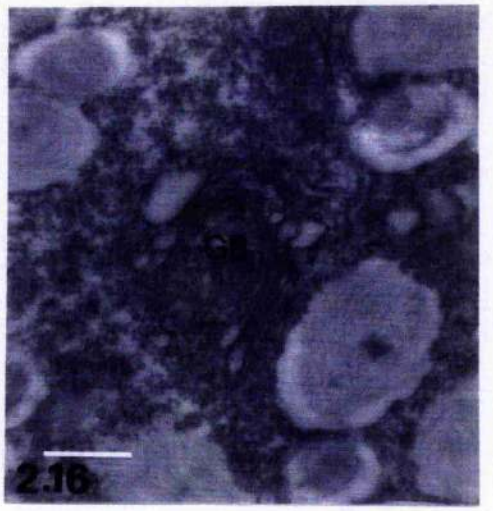

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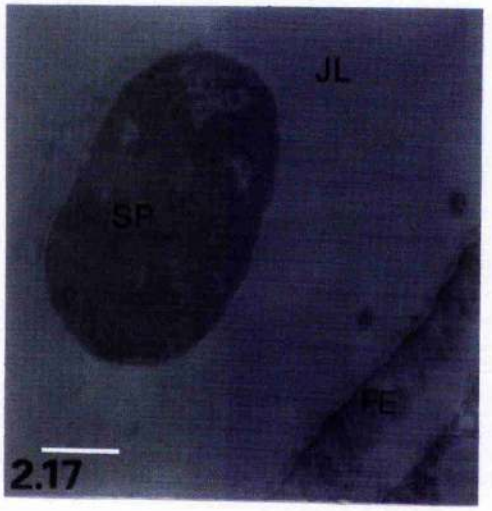

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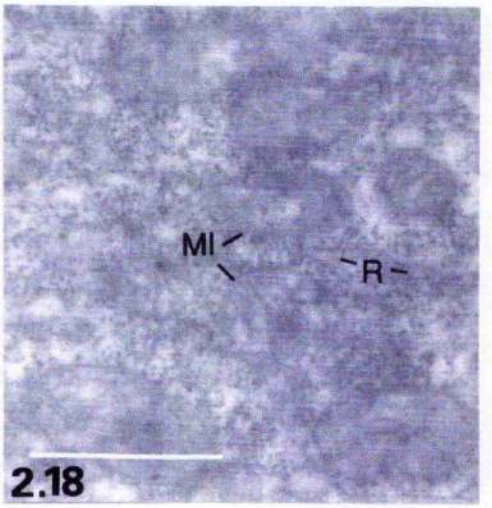

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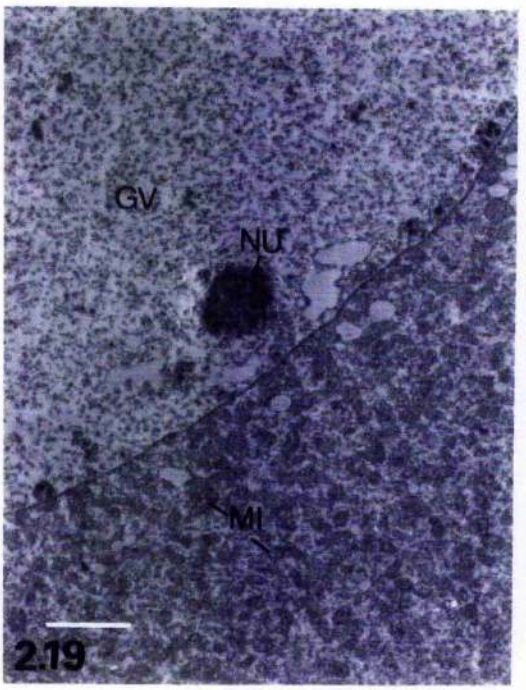

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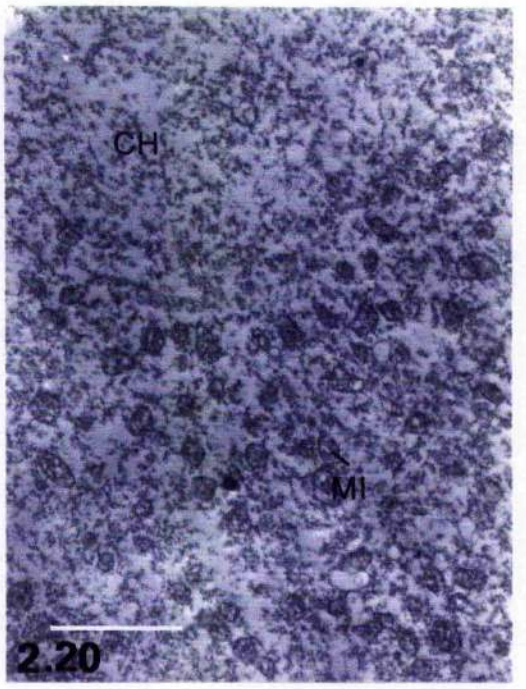


2.20

2.4 DISCUSSION

MAF, as demonstrated here, increases infiltration of the fixatives and provided superior ultrastructural preservation of fertilized *Nereis virens* oocytes. Whereas, conventional fixation results in poor morphological preservation as the extracellular jelly layer acted as a penetration barrier to the fixative chemicals. In addition, MAF considerably shortens fixation time (seconds as opposed to an hour or more) and does not require the removal of the outer gelatinous coating. Furthermore, due to the rapidity of fixation, events such as the moment of cortical granule exocytosis may be frequently fixed and recorded. MAF should also be applicable to other marine invertebrate oocytes and eggs, in addition to other tissues which are difficult to penetrate and for particularly large specimens.

The ultrastructure of fertilization in *Nereis virens* is typical of other nereid species (*N. japonica*, Takashima, 1962; *N. diversicolor*, Pasteels, 1966; *N. limbata*, Fallon and Austin, 1967) whereby fertilization is marked by the mass exocytosis of cortical granules, production of an extracellular jelly coat and elongation of the microvilli. In *N. virens* (Bass and Brafield, 1972) and other nereids (Cross, 1984) the extracellular jelly layer may provide a block to polyspermy. This is thought to occur by the flow of jelly pushing the sperm away or by the sperm becoming embedded and immobilised in the layer (Cross, 1984). Ultrastructural data provided in this chapter support this hypothesis, whereby sperm are located trapped in this outer layer. Nevertheless, the production of this gelatinous coating is not a pre-requisite for successful fertilization and development (Bass and Brafield, 1972). Therefore, polyspermy may not be the prime function and an alternative purpose for this outer coating may well be to protect and cushion the newly formed zygote from the rigours of the external environment.

The inner tubular zone of the vitelline layer in *Nereis virens* oocytes is also found in the oocytes of *N. limbata* (Fallon and Austin, 1967), *N. japonica* (Takashima, 1962) and the Japanese Palolo worm *Tylorrhynchus heterochaetus* (Sato and Osanai, 1983). In addition to providing exit routes for the jelly precursor, the canals in the oocytes of these three species provide the entrance point for the acrosome filament to pass through the vitelline layer (Takashima, 1962; Fallon and Austin, 1967; Sato and Osanai 1983). This could also be the case for *N. virens* although sperm penetration was not examined in this study or by Bass and Brafield (1972).

The research presented in this chapter agrees with the, albeit limited, ultrastructural data gathered by Bass and Brafield (1972). However, these authors state that inseminated *Nereis virens* oocytes develop an elevated fertilization envelope. This was not noted in this study and the ultrastructural data, described here, supports that given for other polychaetes (Cross, 1984). Moreover, Bass and Brafield (1972) declared that all the jelly is exuded within 30 minutes post-insemination however the results of this study showed that cortical granule exocytosis takes up to 100 minutes. This discrepancy could be due to the lower ambient temperature for fertilization used in this study (10°C as opposed to 13°C) which could account, at least in part, to the slower rate of morphological changes. However it should noted that Bass and Brafield (1972) were unable to fix the fertilized oocytes after a certain period of time and therefore how they were able to assess the completion of cortical granule exocytosis is unclear.

Breakdown of the germinal vesicle membrane in *Nereis virens* oocytes is initiated between 80 and 90 minutes post-fertilization at 10°C. A method to score GVBD in oocytes was required for studies described in chapter 6. TEM is a demanding and time-consuming process and therefore inappropriate as a routine scoring method for GVBD and MAF for

fluorescence microscopy was ineffective. However, a method for scoring GVBD in a large number of oocytes was developed. This technique used large, semi-thin sections cut from sample blocks prepared for TEM which were stained and examined by light microscopy. Due to the size of the sections (up to 2 mm square) up to 25 oocytes could be scored for GVBD at one time.

CHAPTER 3

ULTRASTRUCTURE OF MEIOTIC MATURATION IN THE OOCYTES OF *ARENICOLA MARINA*

3.1 INTRODUCTION

In all marine invertebrates, except sea urchins, fully grown oocytes are arrested at prophase I of meiosis. Each of these prophase I oocytes is surrounded by an extracellular coating, most commonly called the vitelline layer (for examples see Chandler and Heuser, 1980; Longo *et al.*, 1982; Schroeder and Stricker, 1983; Schuel, 1984; Longo *et al.*, 1993). Previously, alternative terminology for this layer has included the vitelline membrane (Meijer, 1979b); egg envelope (Eckelbarger and Chia, 1978); surface coat (Gould-Somero and Holland, 1975) and the chorion (Sato and Osanai, 1983). The vitelline layer overlies the oocyte plasma membrane, from which project numerous microvilli (Cross, 1984). Within the oocyte is a large prominent germinal vesicle that accommodates a nucleolus and is surrounded by cytoplasm that contains abundant protein yolk granules, lipid droplets and cortical granules (Cross, 1984). Most marine invertebrate oocytes undergo a second arrest at metaphase, either during the first or second meiotic division (see section 1.2). Release of each meiotic block is always signified by nuclear changes and is often accompanied by alterations in the vitelline layer and the oocyte cortex.

Morphological changes during oocyte maturation are recorded using light, fluorescence, confocal scanning and electron microscopy. Chromosomal and microtubular changes of fertilized *Arenicola marina* oocytes matured *in vivo* and *in vitro* have been described by Watson and Bentley (1998b). Oocytes that have been matured *in vitro* and then fertilized have a significantly slower rate of development than those matured *in vivo* and fertilized (Watson and Bentley, 1998b). This demonstrates that there are subtle differences, hitherto not understood, in oocytes matured by the two methods (Watson and Bentley, 1998b). The oocytes of *A. marina*, both during oogenesis and following spawning, have also been examined using

transmission electron microscopy (TEM) by Rashan (1980). The ultrastructure of the *A. marina* oocytes during the release of the metaphase I block at fertilization has not, until this study, been investigated.

The aim of this part of research is to examine the ultrastructure of *Arenicola marina* oocytes during meiotic maturation using TEM. This will provide a time series of the fine structural changes within the oocyte during release of both the prophase I block (by CMF) and the metaphase I block (by sperm). In addition, the ultrastructure of spawned oocytes (i.e. matured *in vivo*) will be compared to oocytes matured *in vitro,* before and after fertilization. This will allow detection of any morphological differences between oocytes matured using different methods.

3.2 Materials and Methods

3.2.1 Collection and Maintenance of Animals

Populations of *Arenicola marina* are fairly common on the sandy beaches and estuaries around coastlines of the UK. Gravid animals can be collected by digging prior to the natural spawning season. Mature females (i.e. those which will spawn when induced by prostomial homogenate injection) can be obtained from a given population approximately 2-3 weeks prior to the natural spawning season. Males can be induced to spawn experimentally even earlier than this (Pacey, 1991). By collection of *A. marina* from different sites (with different breeding times), mature individuals of both sexes can be obtained for up to 3 months of the year. The populations exploited during this study, and the approximate time of collection, are listed below:

- Kingsbarns, Fife, Scotland (Grid reference [GR] OS-59 604124) October and November
- West Sands, St Andrews, Fife, Scotland (GR OS-59 503185) October and November
- Red Wharf Bay, Anglesey, Wales (GR OS-114 545805) November
- John Muir Country Park, Dunbar, Lothian, Scotland (GR OS-67 643805) November and December

Specimens were collected by digging using a garden potato fork (with flat tines) and the precise digging technique used depended upon the population. Each worm makes characteristic markings upon the sediment surface called the head hole (a circular depression in the sand) and the tail cast (a small pile of excreted sand). In low density populations, worms were dug out individually using these markings to reveal their whereabouts. In high density populations, the animals were collected by "trench digging" which involves continually turning over the surface sediment layer (up to 50 cm) of a large area.

Following collection, the worms were transported back to the laboratory. The animals were sexed by observation of the gametes through the body wall: females contained large numbers of orange coloured oocytes and the males were filled with milky-white seminal fluid. Each specimen was placed into a 250-300 ml plastic or polystyrene tray containing 250 ml of filtered seawater (FSW). The animals were maintained individually within these containers at an ambient temperature of 10°C, with a change of water every few days.

3.2.2 Gamete Collection

Coelomic and Spawned Gametes

Oocytes from within the coelomic cavity were collected using a 1 ml hypodermic syringe, fitted with a 25g needle. The oocytes were washed in twice filtered seawater (TFSW) and used immediately or alternatively stored at 4°C for up to 24 hours before using in experiments.

The induction of spawning in both sexes was achieved by injection of homogenised prostomia, taken from another individual of the same sex (Pacey and Bentley, 1992). The prostomial donor worms were placed under illumination to locate the prostomium, which is a pink-coloured heart shaped structure (approximately 3 mm long) located on the head of the animal. The prostomium was first gripped, using watchmaker's forceps, and then excised using fine iridectomy scissors. The excised prostomia were collected in an Eppendorf microtube containing filtered seawater (250 µl per prostomium) and placed on ice. The tissue was then homogenised using a Soniprep MSE 150 ultrasonic disintegrator and replaced on ice. Each recipient worm was injected with the equivalent of 1 prostomium per individual (approximately 200-250 µl of the homogenate) via a 1 ml disposable hypodermic syringe fitted with a 25g needle.

Following injection, males usually spawned within one hour. Females however, take up to several hours (Pacey and Bentley, 1992).

In Vitro *Matured Oocytes*

Oocytes were matured *in vitro* by incubation with coelomic fluid containing active CMF as described first by Watson and Bentley (1997). To obtain CMF, the CMF-donor females were first injected with homogenised female prostomia (1 prostomium per female). After 30 minutes, a small

sample of coelomic oocytes was withdrawn from the injected worms using a 1 ml hypodermic syringe. The oocytes were placed on a microscope slide before placing a coverslip over them. Using a tissue, the excess water was removed from the coverslip sides before observing for any indications of germinal vesicle breakdown (GVBD) under a light microscope. If no changes were observed, the female was returned and her oocytes re-examined every 30 minutes, for up to 3 hours. Immediately after GVBD had been initiated (as detected by light microscopy) all the coelomic fluid from the female was stripped using a 1 ml hypodermic syringe (fitted with a 25g needle), collected into Eppendorf tubules and then placed on ice. The coelomic fluid was centrifuged for approximately 60 seconds at 13,000 rpm and the supernatant was removed (leaving behind the oocytes) and replaced back on ice.

Immature prophase I arrested oocytes were withdrawn from the coelomic cavity of uninjected females and washed several times in TFSW. Aliquots (3-5 µl) of packed oocytes were placed into a 96 well microtitre plate. Fifty microlitres of the coelomic fluid supernatant (containing active CMF) was added to each oocyte sample and left to mature *in vitro* at 10□C for up to 4 hours.

Fertilized Oocytes

Freshly spawned sperm was mixed with TFSW to give a concentration of 10^6 sperm.ml^{-1} (counted using a haemocytometer). Fifty millilitres of this sperm suspension was added per 450 ml of seawater containing 2 ml of mature (i.e. metaphase I) oocytes, giving a final sperm concentration of 10^5 sperm.ml^{-1}. The oocyte and sperm suspension was mixed briefly and placed at 10°C. After 10 minutes, the seawater was decanted to remove the excess sperm and replaced with fresh TFSW. Approximately 200 µl of fertilized oocytes were then removed from the seawater at 10 minute intervals, and

fixed (see section 3.2.3) for up to 100 minutes to obtain a time course of fertilization.

3.2.3 Fixation for Transmission Electron Microscopy

The following samples were fixed for TEM by conventional chemical fixation:

- Immature prophase I oocytes from the coelomic cavity
- Metaphase I oocytes which had matured *in vivo* and spawned (either spawned spontaneously or following prostomial injection to induce spawning)
- Metaphase I oocytes which had been matured *in vitro* by incubation with CMF
- A time course of fertilized oocytes which had been matured *in vivo* and spawned (either spawned spontaneously or following prostomial injection to induce spawning)
- A time course of fertilized oocytes that had been matured *in vitro* by incubation with CMF.

The unfertilized and fertilized oocytes were fixed for TEM using the fixation protocol described below.

Fixation and Dehydration

The oocytes were rinsed 3 times in TFSW before they were immersed into the primary fixative for 1 hour at room temperature: 2% glutaraldehyde (EM grade, Agar Scientific Ltd), 2% formaldehyde made from paraformaldehyde (EM grade, Agar Scientific Ltd) in a buffer of 0.1M PIPES or 0.1M sodium cacodylate, pH 7, plus 0.1M NaCl, 0.02M $CaCl_2$ and 1%

sucrose. The specimens were then rinsed in the buffer solution (0.1M PIPES or 0.1M sodium cacodylate, pH 7, plus 0.1M NaCl, 0.02M $CaCl_2$ and 1% sucrose) for 15 to 30 minutes and post-fixed in 1% osmium tetroxide (EM grade, Agar Scientific Ltd) in the same buffer for one hour. Prior to dehydration, the specimens were rinsed once more in the buffer for 15 minutes. Dehydration and embedding was completed via an ethanol series at 4□C: 50% (15 minutes), 70% (15 minutes), 96% (15 minutes), 100% (3 x 15 minutes). Half of the specimens were the embedded in LR White medium grade acrylic resin (Sigma) and the remaining samples were embedded in epoxy resin (TAAB Laboratories Equipment), as described in section 2.2.3. Sectioning, section staining, light microscopy and TEM details are also provided in section 2.2.3.

3.3 RESULTS

Prophase I to Metaphase I Transition

The prophase to metaphase I transition is accompanied by breakdown of the germinal vesicle (Figs 3.1 and 3.2). The vitelline layer is a 2 μm thick envelope (Figs 3.3 - 3.4) surrounding the prophase I oocyte and is composed of moderately electron-dense amorphous material (Figs 3.5 - 3.7). The oocyte plasma membrane projects into the vitelline layer in the form of microvilli (Figs 3.5-3.7). The microvilli are forked (Fig. 3.6) and each tip possesses a head made up of several granules that protrude outside the vitelline layer (Fig. 3.7). Directly beneath the oocyte plasma membrane lies a single layer of ellipsoid-shaped cortical granules (Fig. 3.5). In addition to these, the majority of cytoplasm is filled with yolk protein granules and lipid droplets (Figs 3.5, 3.8 and 3.9). Other organelles located in the cytoplasm are ribosomal particles, golgi bodies and numerous mitochondria (Fig. 3.9). The germinal

vesicle possesses a large nucleolus (Fig. 3.9) and is surrounded by a membrane containing many nuclear pores (Fig. 3.10).

Following *in vivo* and *in vitro* meiotic maturation, the oocytes proceed to metaphase I (Fig. 3.2) and a number of notable morphological changes occur. At metaphase I, the microvilli are no longer present and have been withdrawn (Figs 3.11, 3.13 - 3.16), however, the granulated heads of the microvilli remain on the outer surface of the vitelline layer (Fig. 3.16). The cortical granules have expelled their contents (cf. Figs 3.3 and 3.4), creating cavities within the oocyte cortex depicting where these organelles had previously been housed (Figs 3.11, 3.13 and 3.15). At metaphase I, the perivitelline space is wider (Figs 3.11, 3.13 and 3.15) and whether this is due to lifting of the vitelline layer or reduction of the oocyte volume, cannot be ascertained using TEM. Furthermore, the germinal vesicle membrane has broken down, as has the nucleolus (Fig. 3.12).

No ultrastructural differences are observed between oocytes which had matured *in vivo* and subsequently spawned (either by spontaneous spawning or by inducement) with those matured *in vitro* by CMF (cf. Figs 3.13 and 3.14 with 3.15 and 3.16).

Fertilization

Fertilization of the oocytes does not result in any major morphological change in the oocyte cortex. The only significant difference is the separation of the vitelline layer away from the plasma membrane to become the fertilization envelope (Figs 3.4 and 3.17). As with *Arenicola defodiens* oocytes (Meijer, 1979b) the separation of the fertilization envelope is not clearly demonstrated by the electron micrographs. Meijer (1979b) proposed that this was due to the chemical fixation and dehydration procedures used during tissue processing for TEM which induced shrinkage of the outer

layer. The fertilization envelope of *A. marina* oocytes shows no alterations in structure or morphology from the vitelline layer from which it was formed(cf. Figs 3.11 and 3.19).

Following insemination, the slots in the fertilization envelope which housed the microvilli are still present (Figs 3.17 and 3.18), as are the spaces in the oocyte cortex where the cortical granules were previously located (Fig. 3.17). In addition, the membranes of the exocytosed cortical granules ("ghosts") are observed in the perivitelline space (Fig. 3.17). The first polar body is expelled to the perivitelline space at a minimum of 100 minutes post-fertilization (Fig. 3.22).

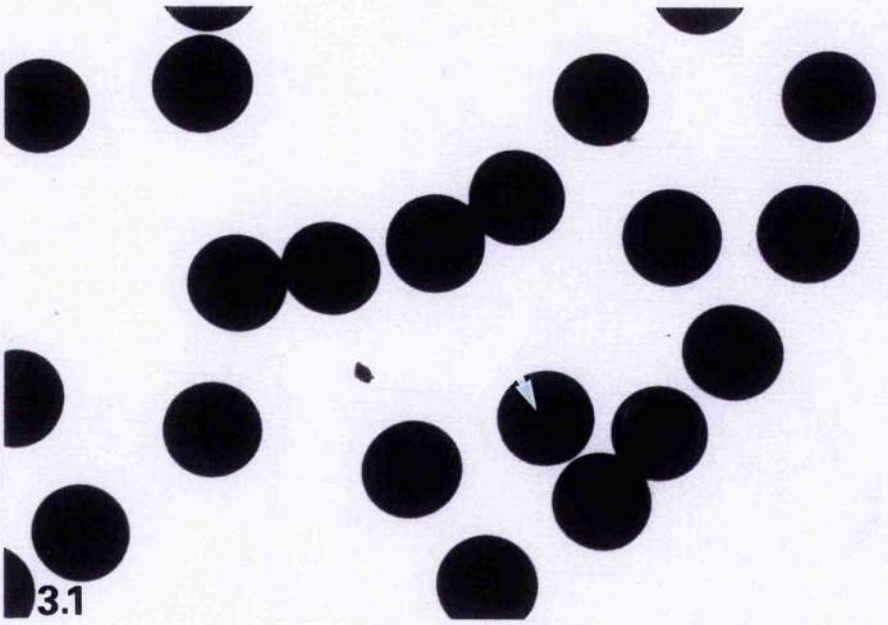
Overall, the fertilized oocytes that had been matured *in vitro* show no ultrastructural differences from those that had matured *in vivo* and spawned (cf. 3.19 and 3.20).

Figure 3.1: Light micrograph of prophase I arrested oocytes with intact germinal vesicles (arrow). Oocyte diameter = 180 μm.

Figure 3.2: Light micrograph of metaphase I arrested oocytes following germinal vesicle breakdown (arrow). Oocyte diameter = 180 μm.

Figure 3.3: Semi-thin section of metaphase I arrested oocyte stained with toludine blue. vitelline layer (arrow). Oocyte diameter = 180 μm.

Figure 3.4: Semi-thin section of fertilized oocyte (60 minutes post-fertilization) stained with toludine blue, showing the separation of the fertilization envelope (arrow). Oocyte diameter = 180 μm.



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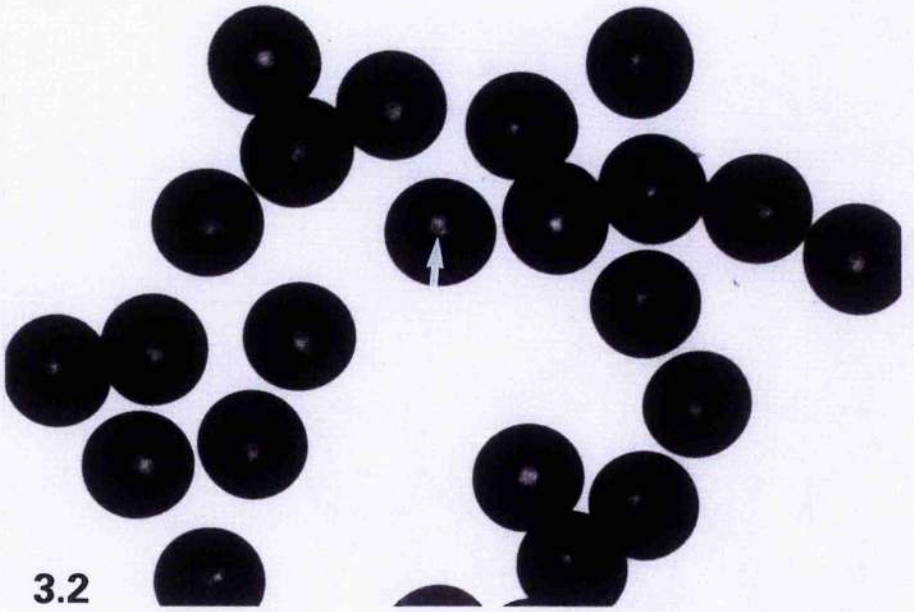

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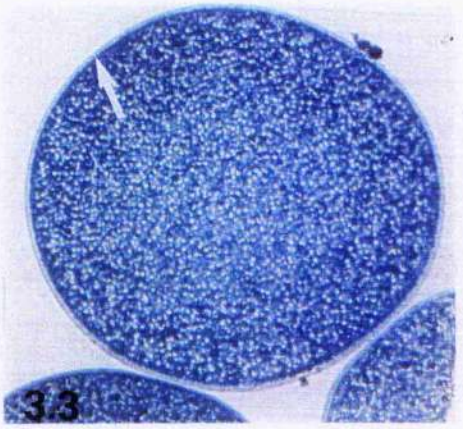

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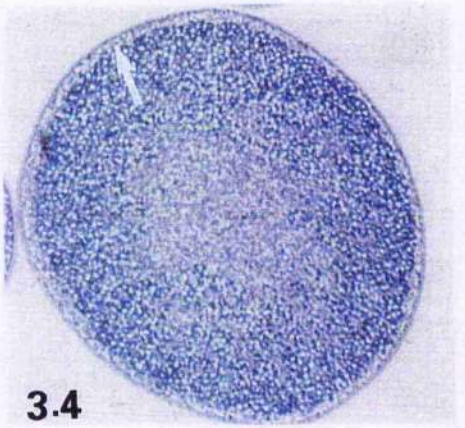


3.4

Figure 3.5: Coelomic prophase I oocyte. The oocyte cortex is lined with a single layer of ellipsoid-shaped cortical granules (CG). The oocyte plasma membrane (OPM) is projected into many microvilli which are embedded within the vitelline layer (VL). protein yolk granules (YG). Scale bar = 0.5 μm.

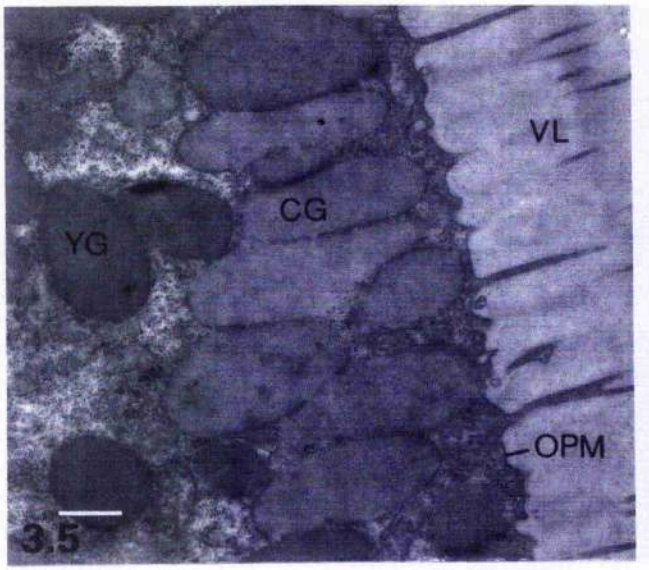
Figure 3.6: Coelomic prophase I oocyte. The microvilli (MV) are forked at their mid-point. vitelline layer (VL). Scale bar = 0.5 μm.

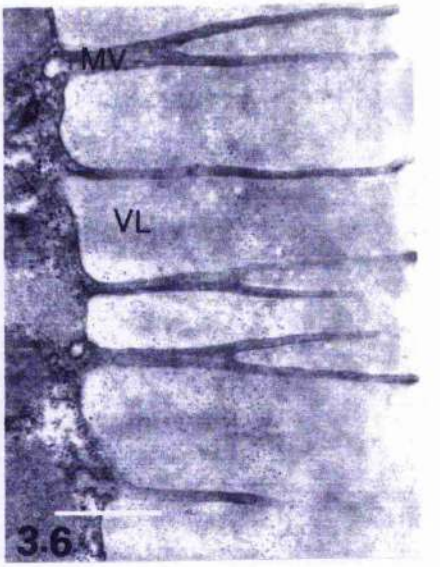
Figure 3.7: Coelomic prophase I oocyte. The microvilli are tipped with granular-type heads (arrow) that protrude outside the vitelline layer. Scale bar = 0.5 μm.

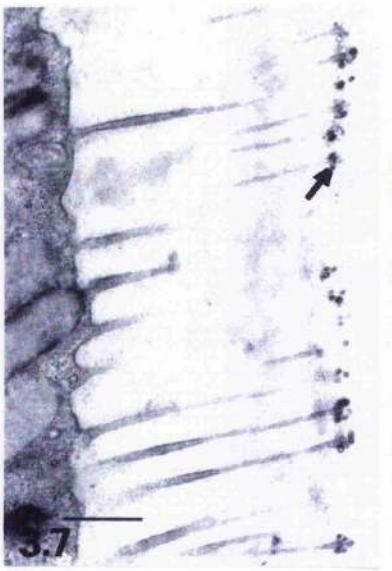
Figure 3.8: Coelomic prophase I oocyte. The oocyte cytoplasm is filled with cortical granules (CG) at the cortex and many protein yolk granules (YG) throughout the cytoplasm. Other organelles found are mitochondria (MI), free ribosomes (arrows) and golgi bodies (GB). Scale bar = 0.5 μm.

Figure 3.9: Coelomic prophase I oocyte. The germinal vesicle (GV) is bounded by an undulating nuclear membrane, and accommodates a large nucleolus (NU). protein yolk granules (YG); lipid droplets (LP). Scale bar = 1 μm.

Figure 3.10: Coelomic prophase I oocyte. The germinal vesicle membrane (GVM) contains many nuclear pore complexes (NP). Scale bar = 0.1 μm.


VL
CG
YG
OPM
3.5


MV
VL
3.6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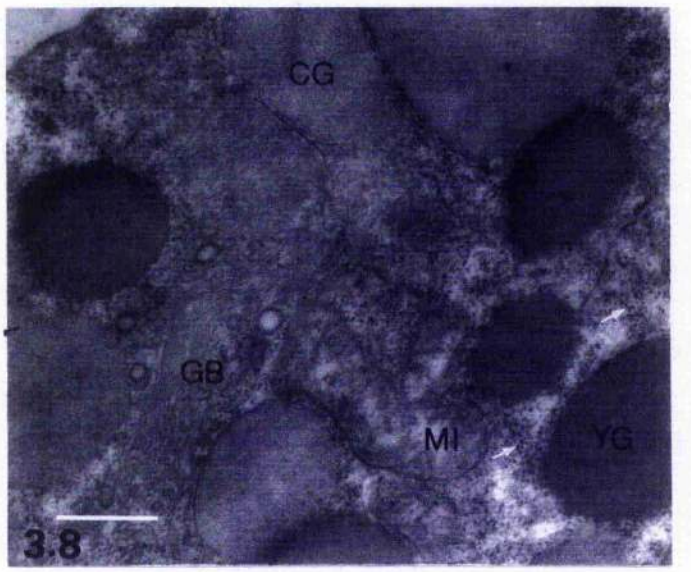
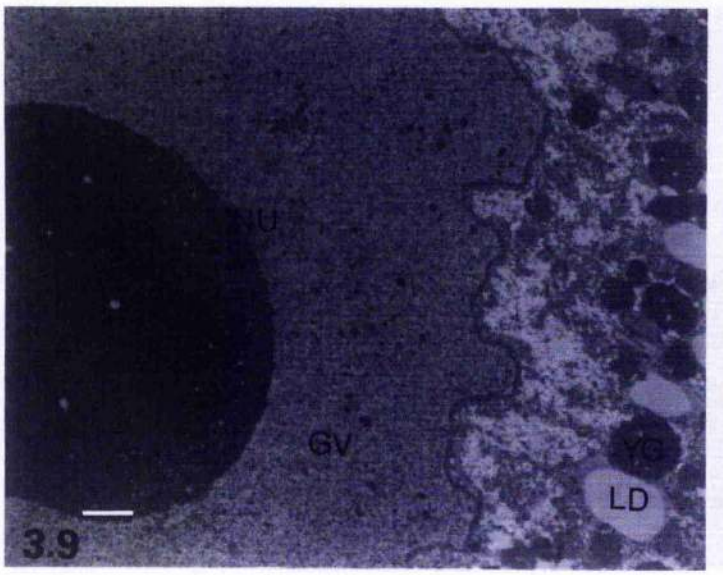

CG
GB
MI
YG
3.8


GV
YG
LD
3.9

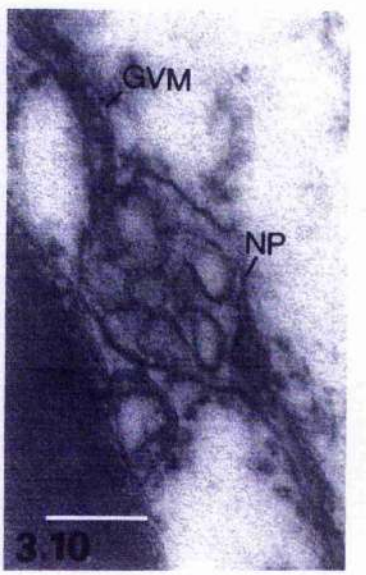

GVM
NP
3.10

Figure 3.11: Spawned metaphase I oocyte. Cortical granules exocytosis has occurred leaving gaps in the cortex (arrows) and the microvilli (MV) have withdrawn from the vitelline layer (VL). A perivitelline space (PVS) has formed between the vitelline layer and the oocyte plasma membrane. Scale bar = 4 μm.

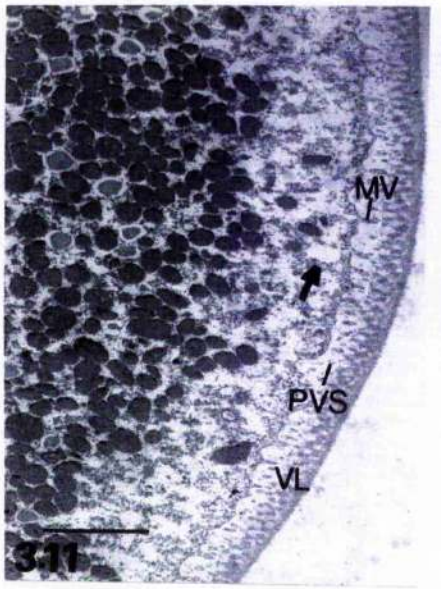
Figure 3.12: Spawned metaphase I oocyte. Germinal vesicle breakdown has occurred releasing the chromatin (CH), which is surrounded by protein yolk granules, lipid droplets and mitochondria. Scale bar = 4 μm.

Figure 3.13 Spawned metaphase I oocyte. Gaps are present in the oocyte cortex depicting where the cortical granules were before exocytosis (arrows). Scale bar = 0.5 μm.

Figure 3.14: Cortex of a metaphase I oocyte, matured *in vivo* and spawned showing the retracted microvilli (MV). Scale bar = 1 μm.

Figure 3.15: Metaphase I oocyte - matured *in vitro* by incubation with CMF Scale bar = 0.5 μm.

Figure 3.16: Cortex of a metaphase I oocyte, matured *in vitro* by incubation with CMF. Note the granules from the microvilli tips have remained on the vitelline layer surface (arrow), following microvilli retraction. Scale bar = 0.5 μm.



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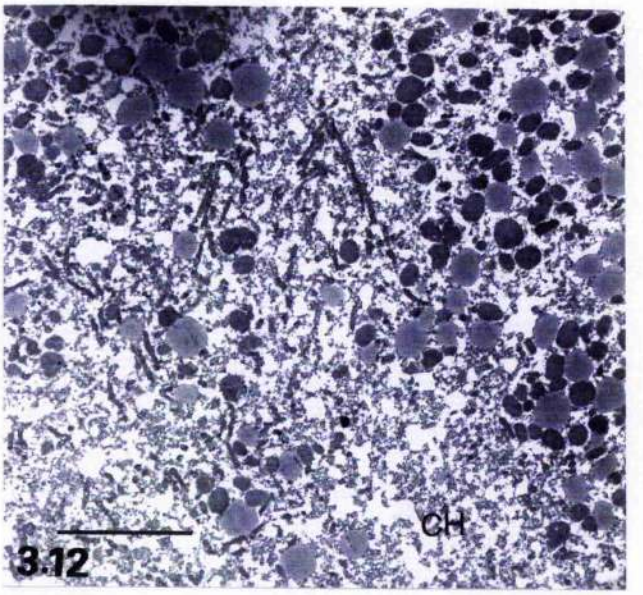

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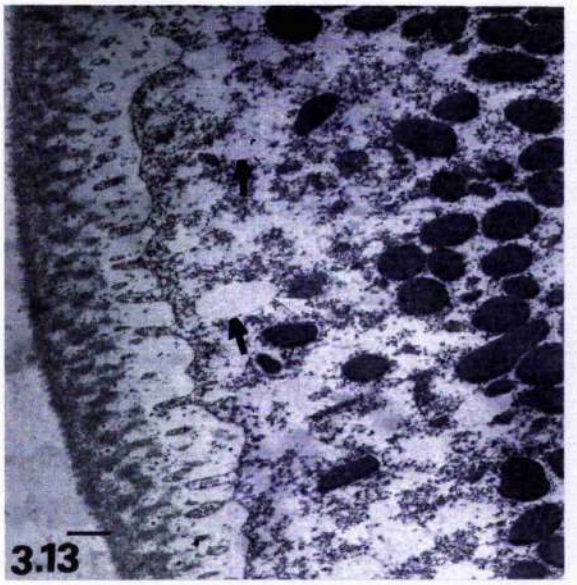
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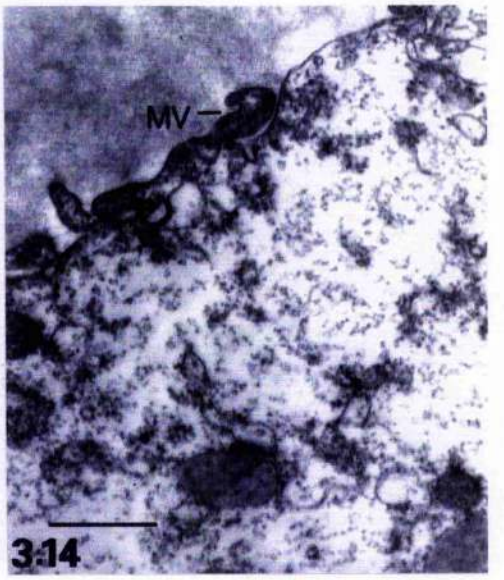

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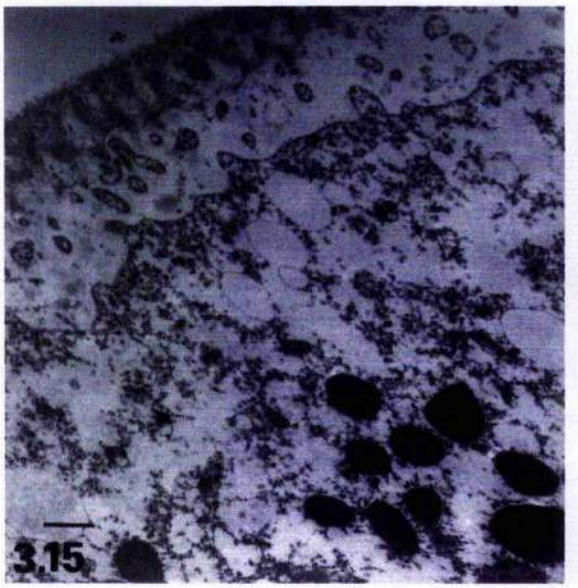
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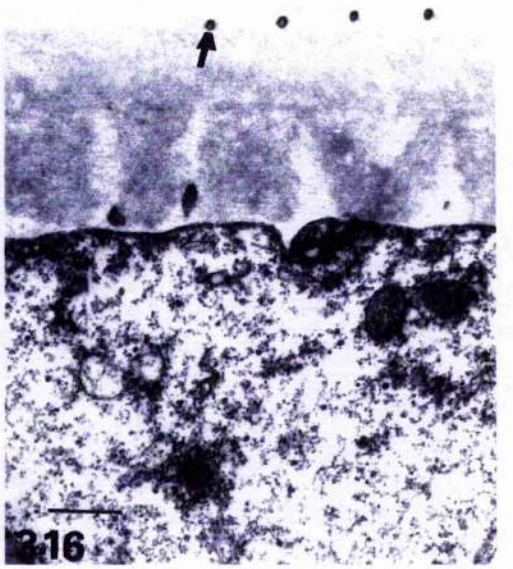

3.16

Figure 3.17: 10 minutes post-fertilization. Cortical granule ghosts (CGG) are located in the perivitelline space (PVS). The vitelline layer is now termed as the fertilization envelope (FE). Scale bar = 1 μm.

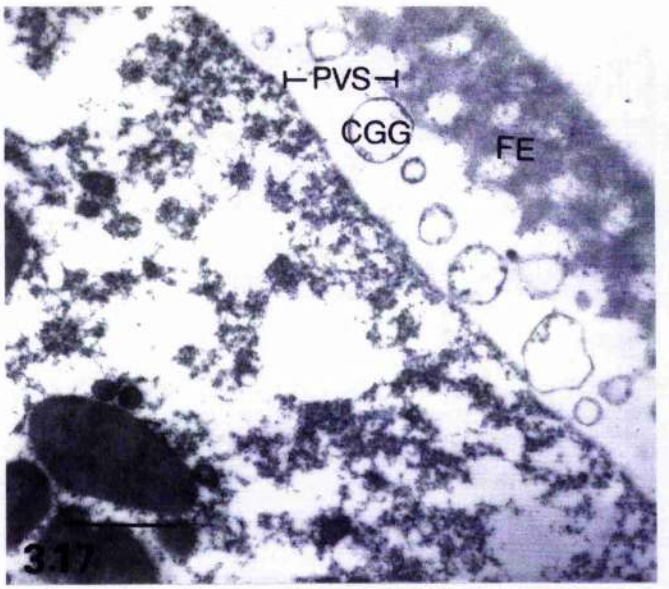
Figure 3.18: 10 minutes post-fertilization. The microvilli (MV) remain retracted and the spaces within the vitelline layer which housed the microvilli can still be observed. Scale bar = 1 μm.

Figure 3.19: 100 minutes post-fertilization. Fertilized oocyte that was matured *in vivo* and spawned. Scale bar = 2 μm.

Figure 3.20: 100 minutes post-fertilization. Fertilized oocyte that was matured *in vitro* with CMF. Scale bar = 2 μm.

Figure 3.21: 100 minutes post-fertilization. Oocyte cortex. Scale bar = 1 μm.

Figure 3.22: 100 minutes post-fertilization. Formation of the first polar body (PB) under the fertilization envelope (FE). Scale bar = 1 μm.



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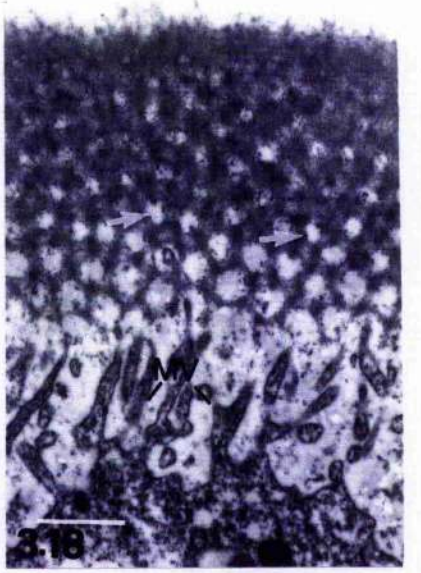

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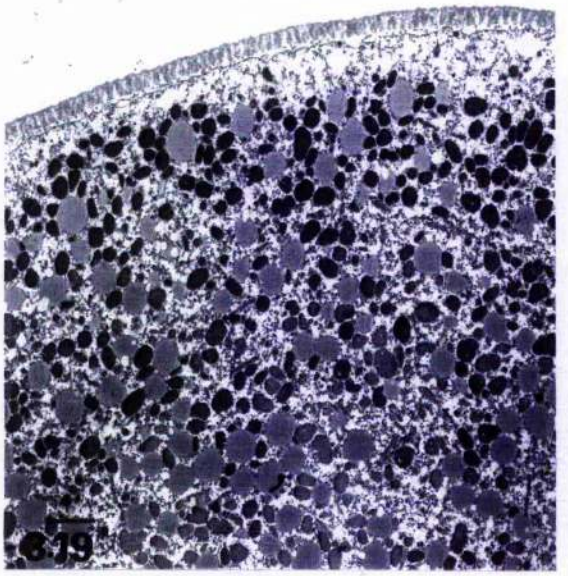
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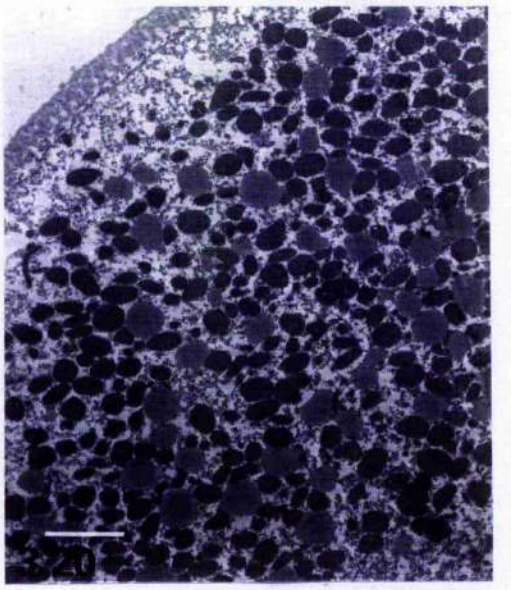
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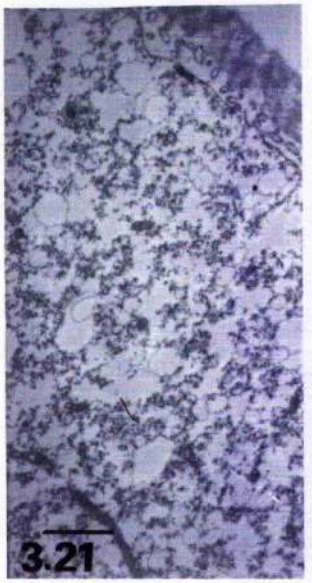
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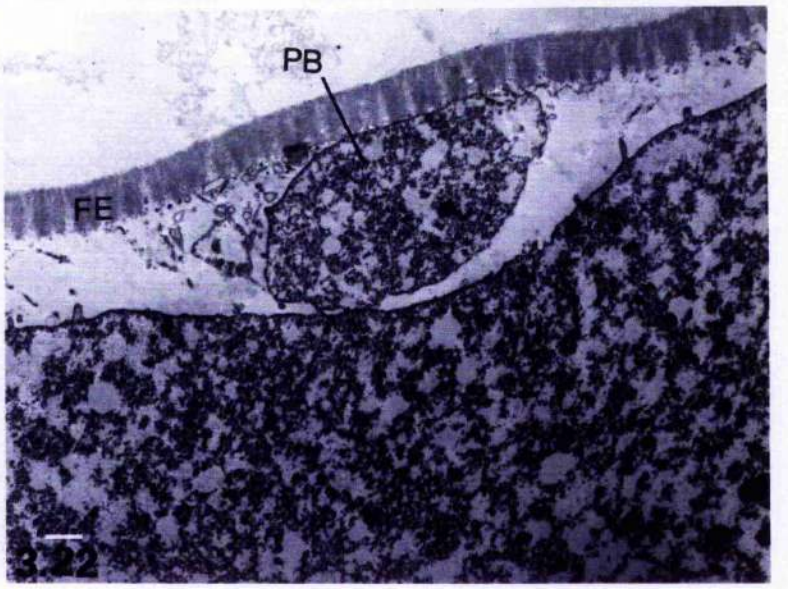


3.22

3.4 DISCUSSION

The ultrastructure of *Arenicola marina* oocytes during the prophase to metaphase I transition has previously been examined by Rashan (1980) and these results here extend these preliminary observations. Changes in *A. marina* oocytes during meiotic reinitiation from the metaphase I block at fertilization had not, hitherto, been described.

In Vivo *versus* In Vitro *maturation*

The ultrastructural changes of *Arenicola marina* oocytes during release from the prophase to metaphase I transition are independent of the method of maturation. Both *in vivo* and *in vitro* oocyte maturation are accompanied by GVBD and marked cortex changes (cortical granule exocytosis and microvillar withdrawal). Oocytes matured *in vitro* have a significantly slower development than those matured *in vivo* and subsequently spawned (Watson and Bentley, 1998b). Watson and Bentley (1998b) discussed the hypothesis that passage through the nephridia during spawning may result in changes to the oocyte, not encountered by the oocytes matured *in vitro*. It is clear, from the research presented in this chapter, that microvillar retraction and cortical granule exocytosis can occur without passage through the nephridia. In addition, there are no differences in the microtubules or chromosomes between *in vivo* and *in vitro* matured oocytes and following fertilization (Watson and Bentley, 1998b). Hence, morphologically, oocytes matured by different methods are identical, before and after fertilization, although the timing of divisions is not. However, a physiological or biochemical change may be induced during *in vivo* maturation and spawning that does not occur during *in vitro* maturation. One proposition is that chemo-attraction (attraction of the sperm to the oocyte) is "switched on" during oocyte maturation *in vivo* (Watson and Bentley, 1998b). If this

hypothesis were correct, this would account for the difference in development rates as the *in vitro* matured oocytes would not posses sperm-attracting properties, time to fertilization would therefore be longer and due to this lag-phase, development would be slower. Data to support this hypothesis reveal that spawned metaphase I oocytes induce directional changes in motile sperm whereas coelomic prophase I oocytes do not (J. Birt, pers comms). This indicates that during maturation and/or spawning, some alteration does occur that provides the oocytes with sperm-attracting ability. The capacity for *in vitro* matured oocytes to induce changes in sperm direction, has yet to be tested.

Cortical Granule Exocytosis

The prophase I to metaphase I transition in *Arenicola marina* oocytes is accompanied by cortical granule exocytosis. The ultrastructure of the prophase I oocytes in the *Arenicola* species studied by Meijer (1979a) is similar to the *A. marina* oocytes in this study. However, in Meijer's study the oocytes do not undergo cortical granule exocytosis during the prophase to metaphase I transition (Meijer, 1979a). These ultrastructural data support the hypothesis that the *Arenicola* species studied by Meijer and Durchon (1977); Meijer (1979a); Meijer (1979b) was not *marina* but was *defodiens* (see also Watson *et al.*, 1998).

As with *Arenicola marina*, cortical granule exocytosis also occurs during spawning in the oocytes of the polychaete *Sabellaria alveolata* (Pasteels, 1965) and the brown shrimp *Penaeus aztecus* (Clarke *et al.*, 1980). Cortical granule exocytosis also occurs during release from the coelom, in the annelid serpulid *Spirobis borealis*, although whether this event occurs prior to fertilization was not determined (King, 1969). The oocytes of *S. alveolata* and *P. aztecus* undergo exocytosis on exposure to seawater (Pasteels, 1965; Clarke *et al.*, 1980). Placing coelomic prophase I oocytes from *A. marina*

into seawater does not induce GVBD, which occurs only when oocytes are exposed to CMF (Watson and Bentley, 1997). The other ultrastructural changes observed during maturation (i.e. cortical granule exocytosis and microvillar withdrawal), however, could be triggered by contact with the seawater as opposed to the direct hormonal effects of CMF alone. During the present study, *in vitro* matured oocytes were first rinsed in seawater before fixation, and hence cannot confirm the above.

Discharge of the cortical granules in the oocytes or eggs of marine invertebrates is more commonly associated with insemination: the polychaetes *Nereis limbata* (Fallon and Austin, 1967), *N. virens* (Brafield and Chapman, 1967) and *Pomatoceros triqueter* (ap Gwynn and Jones, 1971); sea urchins (Pasteels, 1965; Chandler and Heuser, 1981) and starfish (Longo *et al.*, 1982). In nereid oocytes, the material released from the cortical granules forms a jelly layer outside the vitelline layer (Fallon and Austin, 1967; Bass and Brafield, 1972; see also chapter 2). In echinoderms, the exocytosed material is incorporated into the vitelline layer (Longo *et al.*, 1982) and also forms a new "hyaline" layer above the oocyte plasma membrane in sea urchins (Chandler and Heuser, 1980). The function of cortical granule exocytosis, following fertilization, is to release material onto the oocyte or egg surface coats to prevent further insemination (Schuel, 1984). Discharge of the cortical granules in *A. marina* oocytes occurs prior to fertilization and therefore cannot result in a block to polyspermy as the oocyte would never become fertilized.

In the two species in which cortical granule discharge occurs during spawning, exocytosis results in an extracellular jelly coating in the brown shrimp *Penaeus aztecus*, (Clarke, *et al.*, 1980) and in regard to *Sabellaria alveolata* oocytes, the cortical granule contents form a lining underneath the vitelline layer (Pasteels, 1965). In *A. marina* oocytes, no outer jelly layer is

formed, and no extra distinct surface layer was detected in this study. Rashan (1980), however, located the formation of a new moderately electron-dense layer within the perivitelline space in *A. marina* oocytes, in response to exocytosis. One hypothesis is that cortical granule exocytosis may occur during spawning to protect the oocyte, once exposed to the exterior, by adding more material to the vitelline layer. In mollusc oocytes (Allen, 1953; Humphreys, 1967; Dufresne-Dube *et al.*, 1983b; Longo *et al.*, 1993; Désiletset *al.*, 1995) and those of the echiuroid worm *Urechis caupo* (Gould-Somero and Holland, 1975), the cortical granules persist following fertilization, and therefore, as with *A. marina, P. aztecus, S. alveolata* , cannot be responsible for the prevention of polyspermy. The hypothesis that exocytosis can also occur to thicken the outer surface layer for protection is supported by the findings of Humphreys (1967): cortical granule discharge occurs during *Mytilus edulis* embryogenesis, apparently to replace lost surface coat material.

Microvilli Changes

The vitelline layer of *Arenicola marina* prophase I oocytes is typical of this structure observed in other polychaete oocytes (*Marenzelleria viridis* , Bochert, 1996;*Sabellaria alveolata* , Pasteels, 1965; *Pholoe minuta* , Heffernan and Keegan, 1988; *Phragmatopoma lapidosa,* Eckelbarger and Chia, 1978; *P. californica,* Kopp, 1985; *Capitella* species, Eckelbarger and Grassle, 1983; *Hydroides hexagonus* , Colwin and Colwin, 1961; *Pomatoceros triqueter,* ap Gwynn and Jones, 1971; *Nereis virens,* see chapter 2; *Tylorrhynchus heterochaetus,* Sato and Osanai, 1983), consisting of an amorphous layer, with microvilli embedded within, and granules upon the vitelline layer outer surface, that were attached to the microvilli. During the prophase to metaphase I transition in *A. marina* oocytes, the microvilli withdraw from the vitelline layer yet the granular-type heads of the microvilli remain upon the outer surface. Globular- or surface granular-type bodies associated with the

microvilli are common in many polychaete species: *Marenzelleria viridis* (Bochert, 1996)*Sabellaria alveolata* (Pasteels, 1965), *Pholoe minuta* (Heffernan and Keegan, 1988); *Phragmatopoma lapidosa* (Eckelbarger and Chia, 1978); *P. californica* (Kopp, 1985); *Capitella* species (Eckelbarger and Grassle, 1983) *Hydroides hexagonus* (Colwin and Colwin, 1961) *Pomatoceros triqueter* (ap Gwynn and Jones, 1971); *Nereis virens* (see chapter 2); *Tylorrhynchus heterochaetus* (Sato and Osanai, 1983). Analogous to these structures in polychaetes are the tufts situated at the microvilli tips in oocytes of molluscs (Hylander and Summers, 1977) and *Urechis caupo* (Gould-Somero and Holland, 1975). Evidence has been provided that these surface structures upon the vitelline layer could act as sperm binding sites in polychaetes (Colwin and Colwin, 1961; Sato and Osanai, 1983) and molluscs (Hylander and Summers, 1977). This could also be the case for *A. marina*, although actual gamete contact and binding was not examined in this study. No evidence, however, of sperm binding with the surface granules was found in the oocytes of the polychaete *P. californica* (Kopp, 1985). An alternative function for these globular or granular bodies was proposed by Bochert (1996) to facilitate the uptake of material for yolk production

Fertilization Envelope Formation

During spawning, the microvilli of *Arenicola marina* oocytes are withdrawn from the vitelline layer during spawning. This is likely to occur in order to create a perivitelline space for cortical granule discharge and subsequently prepare for the separation of the fertilization envelope from the oocyte plasma membrane. The detachment of the vitelline layer from the oocyte to form the fertilization envelope is well documented in the echinoderms (for examples see Anderson, 1968; Chandler and Heuser, 1980). Elevation of the surface layer in these organisms contributes to the slow block to polyspermy (after initial membrane potential changes which

provide the "fast block" to polyspermy) whereby the sperm are prevented from reaching the oocyte plasma membrane (Schuel, 1984). Furthermore, the fertilization envelope of echinoderms shows marked structural and morphological differences compared with the vitelline layer from which it was formed, becoming significantly harder and thicker (Chandler and Heuser, 1980). As with other polychaetes (Cross, 1984), the separation (i.e. distance of elevation from oocyte plasma membrane) of the fertilization envelope in *A. marina* was considerably less than in echinoderms (Chandler and Heuser, 1980). In addition, the fertilization envelope of *A. marina* oocytes shows little change in its morphology compared to the original vitelline layer, which is also typical of other polychaete oocytes (Cross, 1984). The ultrastructure of the elevated layer in the echiuroid worm *Urechis caupo* oocytes is also unchanged (Gould-Somero and Holland, 1975) as with mollusc oocytes (Hylander and Summers, 1977; Longo *et al.*, 1993; Désilets *et al.*, 1995). In addition to the prevention of polyspermy, it is plausible that the fertilization envelope is thickened in echinoderms to provide extra protection for the developing embryo. The vitelline layer in polychaetes, *Urechis*, and molluscs is relatively thick prior to fertilization (0.5 - 5 μm), when compared to echinoderms (< 0.25 μm in sea urchins), and is therefore likely to offer sufficient protection for the developing zygote without significant hardening and thickening.

CHAPTER 4

ASPECTS OF HORMONE REGULATION CONTROLLING MEIOTIC REINITIATION IN *ARENICOLA MARINA* OOCYTES: ACTIVITY OF PMH AND CMF

4.1 INTRODUCTION

The genus *Arenicola* in Western Europe is comprised of at least two species, *A. marina* and *A. defodiens* (Cadman and Nelson-Smith, 1993). Howie (1963, 1966) was the first to demonstrate that maturation and spawning of female *Arenicola* was under the control of a sex-specific hormone within the brain, the prostomial maturation hormone (PMH). However, unlike *A. defodiens*, the oocytes of *A. marina* do not mature *in vitro* by incubation with PMH (Auckland, 1993; Watson *et al.*, 1998). Auckland (1993) discussed the possibility that progression to metaphase I in *A. marina* oocytes involved another factor, additional to the PMH. This was confirmed by Watson (1997) who discovered that a second substance within the coelomic cavity is responsible directly for oocyte maturation, the coelomic maturation factor (CMF). It is conceivable that CMF and PMH are the same entity, whereby the release of PMH triggers the auto-amplification of more PMH and not a new substance. However, *A. marina* oocytes do not mature *in vitro*, irrespective of PMH concentration (Watson *et al.*, 1998), demonstrating that PMH and CMF are not interchangeable.

Initial characterisation of the 2 hormones shows that PMH storage is confined to the posterior lobes of the prostomium (Howie, 1966), is located in the aqueous fraction of prostomial extracts (Auckland, 1993), and early results indicate it is non-lipid in nature (Howie, 1961b). Active CMF is found in the coelomic fluid and initial investigations into its chemical nature reveals that it is a polypeptide, greater than 30 kDa (Watson and Bentley, 1998a).

The regulation of oocyte maturation in *Arenicola marina* is comparable to that of starfish. Starfish oocyte maturation is initiated by a peptide neurohormone, the gonad stimulating hormone, GSH (Shirai *et al.*, 1986; Kanatani and Shirai, 1971). GSH triggers the production of a second

hormone, 1-methyladenine (1-MeAde) that acts directly on the oocytes to release the prophase I block and induce maturation (Kanatani and Shirai, 1967; Kanatani, 1969; Kanatani *et al.*, 1969). The squamous follicle cells, which surround the starfish oocytes, synthesise and release 1-MeAde (Kanatani and Shirai, 1967; Kanatani, 1969; Kanatani *et al.*, 1969) in response to GSH (Kanatani and Shirai, 1971; Shirai *et al.*, 1986). In many other organisms, including vertebrates, the oocytes are also enveloped in follicle cells and evidence shows that these cells are also often responsible for the production of the Maturation Inducing Factor, MIF (amphibians, Masui and Shibuya, 1987; fish, Nagahama *et al.*, 1995 and see section 1.2 for further details). However, the oocytes of *A. marina* are solitary, free floating within the coelomic cavity (Howie, 1961b) and not associated with any follicle cells that could produce CMF. Nevertheless, there is an additional population of cells within the coelomic fluid of *A. marina* called the "coelomocytes" (Bentley and Pacey, 1989). "Coelomocytes" is the name given to a family of free-floating amoeboid coelomic cells that have a variety of functions. In *A. marina* males, these cells are thought to remove and breakdown any spermatozoa that were not expelled during spawning (Bentley and Pacey, 1989; Pacey and Bentley, 1992a). A phagocytic role for these cells has also been discovered in many other invertebrate species, not necessarily connected with spawning (for examples see Porchethennere *et al.*, 1992; Chia and Xing, 1996). In addition, a sub-population of coelomocytes, called eleocytes, synthesise and supply yolk protein pre-cursors to the vitellogenic oocytes of polychaetes (Fischer and Dorresteijn, 1996) and echinoderms (Cervello *et al.*, 1994).

One hypothesis is that the coelomocytes in the coelomic fluid of *A. marina* females are responsible for CMF synthesis which is released once the trigger from PMH has been received. If this were true, one would expect that incubation of PMH with coelomic fluid containing

coelomocytes would induce the production of active CMF. Initial investigations by Auckland (1993), however, demonstrated that incubation of non-activated coelomic fluid (i.e. not containing CMF) with female prostomial homogenate was ineffective at inducing oocyte maturation. Nevertheless, when Auckland undertook her studies, CMF had not been discovered and therefore no *in vitro* positive control was used. It is possible that the oocytes in her assays may not have been ready to mature.

The aims of the studies reported in this chapter are to begin characterisation of the nature of PMH and to determine the relationship between PMH and CMF activation.

4.2 Materials and Methods

4.2.1 Collection and Maintenance of Animals

For details on collection and maintenance of the worms refer to Chapter 3 (section 3.2.1).

4.2.2 Activity of PMH following Ultrafiltration

A Stirred Ultrafiltration Cell 8050 system (Amicon Inc.) was set up at an ambient temperature of 4°C. The cell was fitted with a 30 kDa Diaflo® membrane Ultrafilter (Amicon Inc.), placed on a magnetic stirrer and connected to a dry nitrogen supply. A thin (4 mm in diameter) piece of silicon tubing was attached to the outflow of the cell and fed into a 15 ml centrifuge tube (placed on ice) for collection of the filtrate. For rinsing, 50 ml of distilled water was added to the cell and filtered through and this process was repeated.

PMH Experiment 1 (PMH 1)

The prostomia of 26 females were removed and homogenised (see section 3.2.2) in 3 ml of twice filtered seawater (TFSW). The female prostomial homogenate was centrifuged (12000 x g for 1-2 minutes) to remove solid waste (cellular fragments, etc.) and then passed through a 0.2 µm filter to remove smaller particulate matter. Three hundred and fifty microlitres of the homogenate supernatant was removed (equivalent to 6 prostomia), made up to 3 ml with TFSW and placed in an Eppendorf tubule on ice ("untreated PMH"). The remaining homogenised extract (equivalent to 20 prostomia) was made up to a total of 5 ml using TFSW and placed into the cell for ultrafiltration.

Running the filter dry can cause the molecules to become attached to the filter and increases protein coagulation making recovery of material very difficult. Ultrafiltration, therefore, was arrested once 4.5 ml of filtrate had been collected, leaving approximately 0.5 ml in the cell. Following this, the filtrate (<30 kDa) was left on ice and 4.5 ml of TFSW was pipetted onto the filter and, after washing, was collected in a 15 ml centrifuge tube and stored on ice (>30 kDa). After rinsing the cell with TFSW, the filtrate (< 30 kDa) was replaced back in the cell, fitted this time with a 10 kDa filter. Following ultrafiltration, the collected filtrate (< 10 kDa) was placed on ice and the filter washed as described before to retrieve the residue (10 - 30 kDa) and placed in a 15 ml centrifuge tube and placed on ice.

42 females were placed individually into 300 ml plastic pots, and covered with 250 ml of filtered seawater (FSW). The positive control treatment worms were injected with female prostomial extract, equivalent to 1 prostomia per female. During the ultrafiltration of the female prostomial extract into size fractions, one would expect to lose some PMH (adhering to the filter and the ultrafiltration cell walls) and for possible degradation

to occur. To counteract this predicted loss of PMH activity, the experimental animals were injected with an equivalent to 2 prostomia per female. The females were injected with the following treatments:

- 10 females were injected with 0.5 ml of the <10 kDa fraction*
- 10 females were injected with 0.5 ml of the 10-30 kDa fraction*
- 10 females injected with 0.5 ml of the > 30 kDa fraction*
- 6 females injected with 0.5 ml of untreated PMH (+ve control)**
- 6 females injected with 0.5 ml of TFSW (-ve control)

* equivalent to 2 prostomia per female
** equivalent to 1 prostomia per female

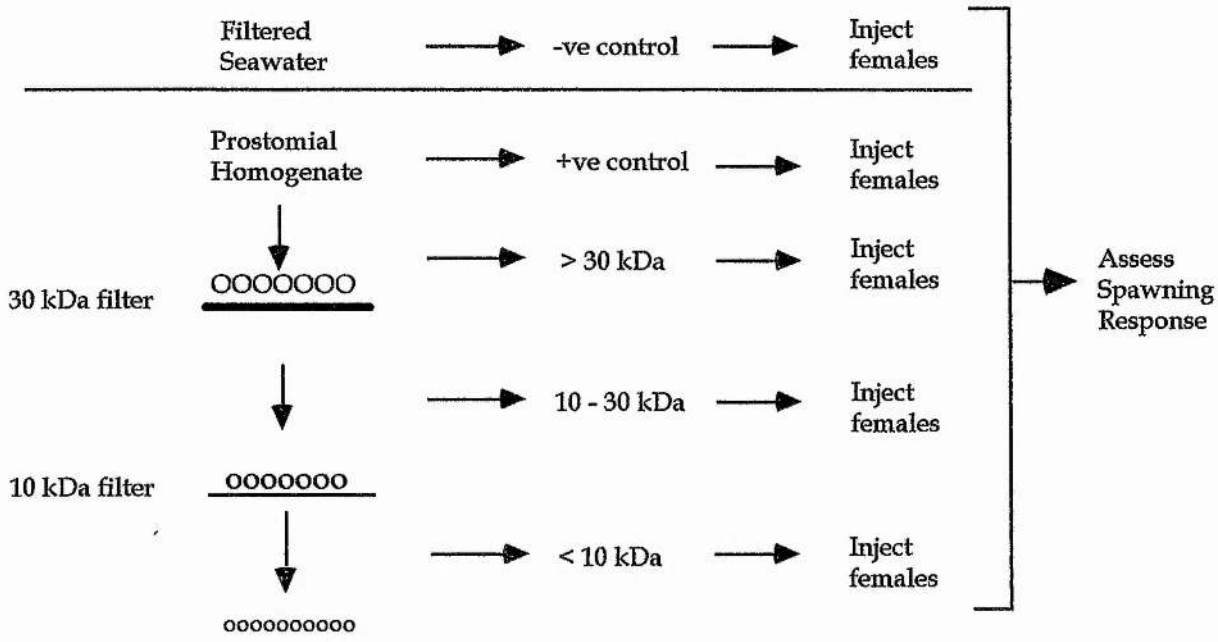


Figure 4.1: Diagrammatic representation of the methodology used to determine the activity of PMH following ultrafiltration.

The worms were left overnight at 10°C and examined for a spawning

response the following morning (approximately 18 hours later). Any spawned oocytes were examined by light microscopical observation to check for GVBD.

PMH Experiment 2 (PMH 2)

The methodology for this second experiment was essentially the same as given in PMH1 but with the following modifications. Each treatment group consisted of 8 females and a total of 28 prostomia were used for injection. Prostomial homogenate equivalent to 12 prostomia was injected into the positive controls (1.5 prostomia per female) and 16 prostomia was used for ultrafiltration to be injected into the experimental animals (2 prostomia per female). In contrast to the previous experiment (PMH1), a mixture of freshly excised prostomia (n = 19) and frozen prostomia (n=9), removed previously from females and either stored at -20°C or -70°C, was used. Additionally, following ultrafiltration through the 30 kDa filter and collection of the < 30 kDa filtrate, 10 ml of TFSW was added and run through the cell. This filtrate was not collected but this step was carried out so that any molecules smaller than 30 kDa would be thoroughly washed through and not collected on the filter. Following this, the residue from the filter was resuspended as described before and collected. All other methods used are the same given for PMH1.

4.2.3 Activation of CMF

Oocyte Maturation Assay

In vitro maturation with coelomic fluid containing active CMF rarely produces 100% GVBD in a sample of oocytes (Watson and Bentley, 1997; Watson *et al.*, 1998). Moreover, in some cases the oocytes do not respond at all, even though the CMF was known to be active (pers. obs.). This is because females are used during experimentation, prior to their

natural spawning season and hence the oocytes may not be ready to respond to CMF. For these experiments, therefore, the oocytes were first assessed for their ability to mature. A small sample (3-5µl) of oocytes was taken from each potential donor female and placed separately in the compartments of a 96-well plate. Coelomic fluid containing active CMF (see section 3.2.2) was pooled from 3 females and 50 µl was aliquoted into each well containing the oocytes. The oocytes were left at 10°C and scored for GVBD after 4 hours. Females were only used in the following experiments if a substantial proportion of their oocytes (approximately >30%) underwent GVBD.

CMF Activation Experiment 1 (CMF 1)

For this experiment, 7 females were used and coelomic fluid containing active CMF was obtained from a separate group of 3 females (see section 3.2.2), pooled and used for the positive control.

The prostomium was removed from the first donor female (DF1) and placed in 500 µl of filtered seawater. All the coelomic fluid and oocytes were stripped from DF1 and placed into Eppendorf tubules and centrifuged at 12,000 x g for 1 minute. The inactive coelomic fluid (i.e. does not contain active CMF) was separated from the oocytes and both oocytes (in TFSW) and fluid were kept on ice. 10 x 5µl of DF1 oocytes were aliquoted into a 96-well plate. A different treatment was added to each oocyte sample in duplicate:

i) 100 µl of TFSW (-ve control)

ii) 100 µl of inactive coelomic fluid

iii) 100 µl of female prostomial homogenate

iv) 100 µl of female prostomial homogenate + 100 µl of inactive coelomic fluid

v) 50 µl of active coelomic fluid containing CMF (+ve control)

The above procedure was repeated for the other 6 donor females.

CMF Activation Experiment 2 (CMF 2)

If one assumes that PMH triggers CMF activity from within the coelomic fluid, then the amount of fluid required per unit of CMF activity can only be estimated. The previous experiment (CMF1) used one volume of female prostomial homogenate (at a final concentration of 1 prostomia.ml^{-1}) with one volume of inactive coelomic fluid, yet incorrect ratios could lead to negative results. In this experiment, therefore, whole worm extracts were used. The prostomium was removed from each female (n=6) and homogenised individually in 100 µl of TFSW (see section 3.2.2). The entire contents (oocytes, coelomic fluid and blood) were removed from the coelomic cavity of the 6 worms and placed into six 10 ml glass vials. Small samples of oocytes were removed from each, washed and used for the positive (incubation in active CMF) and negative (incubation in TFSW) controls. The respective homogenised prostomia were added to the entire coelomic cavity content (oocytes, coelomic fluid and blood) of the appropriate female and left at 10°C. The oocytes were checked at 2, 4, 6 and 8 hours later and scored for GVBD.

4.3 RESULTS

4.3.1 Activity of PMH following Ultrafiltration

PMH Experiment 1 (PMH1)

Of the females injected with prostomial fractions above 10 kDa and females injected with untreated PMH (positive control), 50 % and 33%

spawned respectively (Fig. 4.2). All the spawned oocytes had undergone GVBD, representing a true spawning response. No spawning occurred in the negative control or as a result of injection of the prostomial fraction smaller than 10 kDa (Fig. 4.2). For statistical analysis, the data (in proportions) were arc-sine transformed. Analysis (r x c test for independence using a G-test) shows that there was a significant difference between the treatments ($p < 0.01$). Further analysis using a post-hoc Tukey multiple comparison for proportions revealed that there is no significant differences in spawning response when injected with the "10 - 30 kDa" prostomial fraction, the ">30 kDa" fraction or the positive control. The differences, however, between these 3 treatments and either the negative control or injection of the "< 10 kDa" fraction were highly significant ($p < 0.001$).

PMH Experiment 2 (PMH2)

The results from PMH1 indicate strongly that the molecular weight of PMH is greater than 10 kDa, yet both the "10-30 kDa" and "> 30 kDa" fractions gave positive spawning responses. This experiment was repeated in an attempt to clarify these results (i.e. which molecular size fraction PMH belonged to) and some adjustments were made to improve the methodology (see section 4.2.2). In this experiment (PMH2) only the females treated with the positive control spawned (2 from 8). There was no significant difference between the spawning response of the different treatments (r x c test of independence using a G-test, $p > 0.05$).

4.3.2 Activation of CMF

In experiments CMF1 and CMF2, GVBD was only initiated by the positive control, i.e. the addition of coelomic fluid containing active CMF (see tables 4.1 and 4.2). The negative control and all other treatments

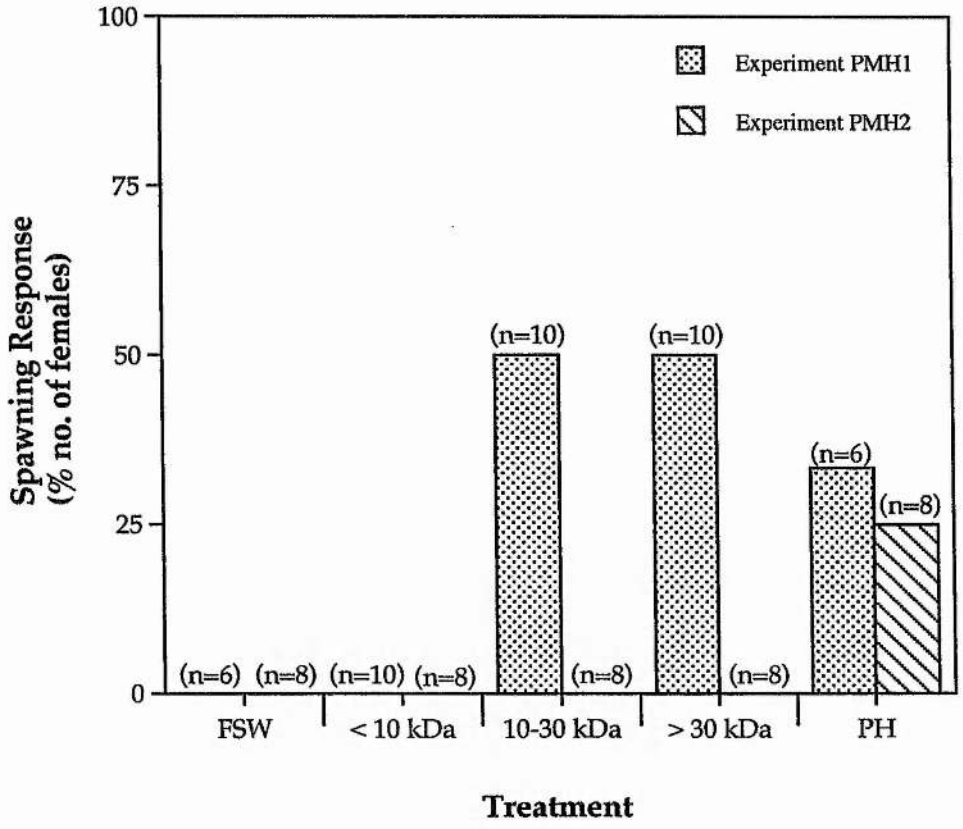


Figure 4.2: Percentage number of females spawning in response to injection with different size fractions of the prostomial homogenate (< 10 kDa; 10 - 30 kDa; > 30 kDa) and control treatments (FSW - filtered seawater; PH - prostomial homogenate). n = no. of females per treatment.

induced no changes in the meiotic arrest of the oocyte. During experiment CMF2, after several hours incubation in prostomial homogenate and coelomic fluid, many of the oocytes showed marked degeneration, but oocytes incubated in the TFSW did not.

Mean Percentage GVBD*

Female Number	TFSW	FPH	Inactive CF	FPH+ Inactive CF	Active CF
1	0	0	0	0	61
2	0	0	0	0	62
3	0.01	0	0.02	0	68
4	0	0	0	0	43
5	0	0	0	0	59
6	0	0	0	0	32
7	0	0	0	0	57

* 3 x 50 oocytes counted per sample

Table 4.1: Results from CMF1. Mean percentage GVBD following incubation of the oocytes in various treatments (TFSW - twice filtered seawater; FPH - female prostomial homogenate; Inactive CF - inactive coelomic fluid; Active CF - coelomic fluid containing active CMF)

	Mean Percentage GVBD*		
Female Number	TFSW	Inactive CF + FPH	Active CF
1	0	0	53
2	0	0	59
3	0	0	48
4	0	0	32
5	0	0	41

* 3 x 50 oocytes counted per sample

Table 4.2: Results from CMF2. Mean Percentage GVBD following incubation of the oocytes in various treatments (TFSW - filtered seawater; FPH - female prostomial homogenate; Inactive CF - inactive coelomic fluid; Active CF - coelomic fluid containing active CMF).

4.4 Discussion

The technique of ultrafiltration assumes that the molecule is spherical, which is often not the case. Hence, whether a substance passes through a filter is not only dependent upon the size but also the shape. Nevertheless, ultrafiltration is still a useful method in indicating the size characteristics of the substance in question, and provides is a useful step for the initial stages of purification. The results from the first experiment (PMH1) conducted on the ultrafiltration of prostomial extract indicate strongly that PMH in *Arenicola marina* females has a molecular mass greater than 10 kDa. Both the "10-30 kDa" and "> 30 kDa" prostomial fractions, however, result in a significant spawning response. The ambiguity in these results could be due to the active factor being greater than 30 kDa but due to membrane damage some passed through. Alternatively, PMH is equal to or near to a molecular mass of 30 kDa, and therefore some PMH passed through and some

remained on the filter. A third possibility, is that PMH is less than 30 kDa but some of the active factor remained in the filter residue, thus becoming incorporated into the ">30 kDa" fraction. Further experimentation is required using gel filtration and SDS PAGE to conclude the true molecular size of PMH.

The second ultrafiltration experiment (PMH2) that was carried out was unable to confirm the results of the first (PMH1). None of the worms injected with different molecular size fractions of prostomial extract produced a positive spawning response and only 25% of the females injected with the positive control spawned. The reason for such low PMH activity may have been due to the prostomia that was homogenised and used for injection. Thirty percent of the prostomia used were dissected from females at an earlier date and had been preserved at -20°C or -70 °C. It is possible that the frozen prostomia (excised up to 2 weeks prior to the experiment, and therefore up to 3 weeks prior to the natural spawning season) contained very low levels of PMH and hence the concentration of the active factor in the pooled prostomial homogenate was below the threshold required to induce spawning.

The release of PMH triggers the production of CMF activity (Watson and Bentley, 1997; Watson *et al.*, 1998) but the precise pathway to activation has yet to be ascertained. Indirect evidence gathered by Watson (1996) suggests that CMF is synthesised prior to spawning and not synthesised *de novo*. In either case, the location of CMF synthesis and/or site of activation or release will aid further understanding of the hormonal cascade leading to meiotic reinitiation. Active CMF is found within the coelomic fluid, and one hypothesis is that it is synthesised by the coelomocytes (before or at the time of spawning) and released once the PMH signal is received. Alternatively, CMF could exist prior to spawning as an inactive "pre-CMF" form within the

coelomic fluid and then transformed to the active form. In either case, incubation of prostomial homogenate with coelomic fluid (containing coelomocytes) should induce CMF activity *in vitro*. The investigation reported here demonstrates that this was not the case. One explanation for these negative results is that CMF is synthesised and/or stored at an alternative location (e.g. a specific gland, the body wall, the gut cells or within the blood vessels). On the other hand, PMH may induce CMF activity within the coelom (i.e. in the coelomocytes or fluid), but due to other factors it was not activated *in vitro*. For example, this could be as a result of catabolic enzymes within the body fluids that caused the breakdown of active CMF before it induced oocyte maturation. To support this suggestion, the oocytes incubated in coelomic fluid, blood and prostomial extract showed marked degradation as opposed to those in TFSW, indicating that catabolic enzymes were present. A further possibility is that PMH does not induce CMF activity directly, but activates an intermediate pathway, that was not established *in vitro*.

One potential intermediate pathway is the nervous system. Does the release of PMH trigger a second neuroendocrine step which then triggers CMF activity? Previous work shows that decerebrated females will spawn in response to prostomial injection (Howie, 1963). Therefore, if an intermediate step exists between the release of PMH and activation of CMF, then the results from the investigation of Howie (1963) indicate that the prostomium is not involved.

CHAPTER 5

CHEMICAL INDUCTION OF MEIOTIC REINITIATION IN *ARENICOLA MARINA* AND *A. DEFODIENS* OOCYTES

5.1 INTRODUCTION

Two possibilities exist for sperm initiated resumption of meiosis (Swann *et al.*, 1994). The first is that sperm binds with a receptor upon the oocyte plasma membrane which in turn triggers the cascade leading to meiotic reinitiation (Abassi and Foltz, 1994; Shilling *et al.*, 1994). Alternatively, there is evidence that the sperm first interacts with the oocyte plasma membrane and then releases a diffusable proteinaceous product into the oocyte cytoplasm, that activates the pathway to meiotic resumption (Stricker, 1996, 1997). The "second messenger" is the name given to the intracellular factor responsible for the transduction of the MIF (maturation inducing factor) signal to MPF (M-phase promoting factor) activation. The second messenger in fertilization induced meiotic reinitiation is calcium (see Whitaker and Swann, 1993, for review). Studies of the role of calcium at fertilization have predominantly focused upon deuterostome oocytes: sea urchin (see Shen, 1995, for review); ascidian (Speksnijder *et al.*, 1990); fish (Gilkey *et al.*, 1978); frog (Kume *et al.*, 1997) and mammal (Tombes *et al.*, 1992). The role of calcium in protostomes at insemination is not as thoroughly studied, and largely been confined to the Mollusca (for example see Deguchi and Osanai, 1994a). Fertilization in both protostomes and deuterostomes triggers an increase in intracellular calcium levels, that in turn trigger meiotic reinitaition and oocyte activation (see Whitaker and Swann, 1993, for review). One hypothesis is that the cytosolic rise in calcium ions at fertilization is derived from intracellular stores in deuterostome oocytes and from the external medium in protostome oocytes (Jaffe, 1983).

Hormone induced reinitiation of meiosis begins at the oocyte plasma membrane via G-protein receptors (Hoshi *et al.*, 1992; Tadenuma *et al.*, 1992; Jaffe *et al.*, 1993; Gobet *et al.*, 1994). The second messenger that transduces this signal to the cytoplasm, however, has yet to be elucidated. Among the

potential signals, calcium (see section 1.3.2), pH (see section 1.3.3), trypsin and the hydroxyeicosatrenoic (HETE) acid (see section 1.3.6) mediated pathways have all been proposed.

The preferred method to commence investigation of the second messenger during meiotic reinitiation is to test the affect of specific chemical agents, with known pharmacological actions, on the oocytes. Parthenogenic activation of *Arenicola marina* oocytes has not been investigated but Watson *et al.* (1998) attempted to mimic CMF-induced meiotic maturation by using chemicals known to induce the release of calcium ions primarily from intracellular stores (Watson, 1996): ionophore A23187, tetracaine, procaine, propranolol, oxproprenol and lanthum chloride. All of these chemicals were unsuccessful indicating that CMF-induced maturation is calcium-independent (Watson *et al.*, 1998). Nevertheless, during *in vivo* oocyte maturation in *A. marina*, a calcium rise may initiate meiosis, but is derived not from intracellular stores, but taken from the external medium.

The aims of the studies reported in this chapter are to test the effect of a number of treatments for their ability to mimic CMF-induced meiotic maturation in *Arenicola marina* oocytes using the following treatments: an influx of calcium from the exterior (using excess potassium ions); arachidonic acid and pH changes. In addition, the effect of calcium ionophore A23187 and arachidonic acid on oocyte maturation in *A. defodiens* are to be tested. Finally, different treatments are to be used in an attempt to activate *A. marina* oocytes parthenogenically (i.e. induce release from the metaphase I block without sperm).

5.2 MATERIALS AND METHODS

5.2.1 Treatment Solutions

The following experimental treatment solutions were investigated.

Calcium-free SW: Calcium-free seawater was made up using MBLs formula (Cavanaugh, 1956): 25.53g NaCl, 0.67g KCl, 4.66g $MgCl_26H_2O$, 6.29g $MgSO_47H_2O$, 0.18g $NaHCO_3$ in 1 l of distilled water.

CMF (coelomic maturation factor): Used for the positive control for *in vitro* incubation of *Arenicola marina* prophase I oocytes: coelomic fluid containing active CMF was obtained from 1 to 3 females and pooled (see section 3.2 for details).

Ethanol: A 1% stock solution of ethanol was made up in TFSW and diluted to the appropriate concentration using TFSW.

Excess ammonium ions: A solution of NH_4SO_4 was made up to the appropriate concentration using TFSW.

Excess calcium ions: A stock solution of 0.34M $CaCl_2$ (isotonic with seawater) was made up with distilled water and diluted to the appropriate concentration using TFSW.

Excess potassium ions: A stock solution of 0.52M KCl (isotonic with seawater) was made up with distilled water and diluted to the appropriate concentration using TFSW.

Female prostomial homogenate: Used for injection to induce spawning in *Arenicola marina* (see section 3.2). Used for the positive control for *in vitro* incubation of *Arenicola defodiens* prophase I oocytes: the prostomia were removed and homogenised in TFSW before being diluted to 0.5 prostomia.ml^{-1} using TFSW.

Ionophore A23187: A stock solution of 10^{-3}M ionophore A23187 was made up in ethanol and diluted to the appropriate concentration using TFSW.

Seawater with different pHs: The pH of TFSW was decreased by the addition of 1M HCl and increased by the addition of 1M NaOH solution.

TFSW + Sperm: 2 or 3 males were injected with male prostomial homogenate (1 prostomium per male). After 1 to 2 hours (males usually spawn 1 hour post-injection) the freshly spawned sperm was collected and diluted with TFSW to obtain a concentration of approximately 10^{-5}M (counted using a haemocytomter).

TFSW: Twice filtered seawater.

Trypsin: Type III bovine pancreas trypsin was used and dissolved in TFSW to the appropriate concentration.

5.2.2 Activation of Prophase I Oocytes - *A.marina* and *A. defodiens*

Arenicola defodiens

Oocytes were removed from 3 females as described in section 3.2.2 and 20 µl aliquots were placed individually into a 96 well plate. The oocytes for each female were tested separately, i.e. each treatment at each concentration was tested on 2 samples of oocytes from each female. Two hundred microlitres of each treatment solution (ionophore A23187 and arachidonic acid at 10^{-7}M, 10^{-6}M and 10^{-5}M respectively) was added individually to the oocyte samples. The oocytes were also incubated in TFSW and 1% ethanol for the negative controls and in female prostomial homogenate for the positive control. Incubation time was 4 hours at 10°C, after which time the oocytes were scored for germinal vesicle breakdown, GVBD (2 x 50 oocytes counted per sample).

Arenicola marina

Two groups of 3 females were used. The oocytes were removed from one set of 3 females (batch 1) as described in section 3.2.2, and then washed in TFSW before aliquoting the oocytes (20 µl) into wells of a 96 well plate. Two hundred microlitres of treatment solution, at the required concentration, was then added to 2 samples of oocytes from each female. The following treatment solutions were tested: excess potassium ions (final concentrations = 52mM KCl, 104mM KCl, 260mM KCl); excess calcium ions (final concentrations = 68mM $CaCl_2$, 170mM $CaCl_2$); TFSW with modified pH (pH 6, pH 7, pH 8, pH 9, pH 10, p 11, pH 12) and excess ammonium ions (final concentrations = 10mM NH_4SO_4, 50mM NH_4SO_4). The oocytes were withdrawn from the second set of 3 females (batch 2) and incubated in arachidonic acid (10^{-7}M, 10^{-6}M, 10^{-5}M). For the negative controls, oocytes were incubated in TFSW or 1 % ethanol and the positive control oocytes

were incubated in CMF. After 4 hours at an ambient temperature of 10□C, the oocytes were scored for GVBD (2 x 50 oocytes per sample).

5.2.3 Activation of Metaphase I Oocytes - *A. marina*

Arenicola marina

Metaphase I oocytes were obtained from females that had spawned spontaneously or had been induced to spawn by female prostomial injection (see section 3.2.2 for details). The spawned oocytes were first checked for GVBD before being used for experimentation. The oocytes from 3 females were used per batch of chemicals to be tested. The oocytes from each female were tested separately, 2 samples of oocytes from each female per treatment concentration. The gametes were withdrawn from the first set of 3 females (batch 1), washed and then 50 µl aliquots were placed in the compartments of a 24 well-plate. Two millilitres of TFSW containing excess potassium ions (final concentration = 52mM KCl; 104mM KCl; 260mM KCl) or excess calcium ions (final concentration = 68mM $CaCl_2$; 170mM $CaCl_2$) was added to the oocytes for each female, in duplicate. The oocytes of another set of 3 females (batch 2) were incubated in TFSW with modified pH (pH 6, pH 7, pH 8, pH 9, pH 10) and excess ammonium ions (final concentration = 10mM NH_4SO_4, 50mM NH_4SO_4). Two millilitres of ionophore A23187 (10^{-7}M, 10^{-6}M, 10^{-5}M) were added to the oocytes of a third batch of 3 females. In each case, the following control solutions were used: TFSW (negative control) and TFSW plus active sperm (positive control) and an additional negative control for the ionophore treatment (1%, 0.1% and 0.01% ethanol solutions). In addition, oocytes from another 3 females were incubated in calcium-free seawater and calcium-free seawater plus sperm. All the oocytes were incubated at 10°C for 2 hours and then scored for activation, which was assessed by the presence of a raised fertilization envelope (2 x 50 oocytes counted per sample).

5.3 RESULTS

5.3.1 Activation of Prophase I Oocytes - *A. marina* and *A. defodiens*

Arenicola defodiens

The oocytes from 1 of the 3 females underwent spontaneous GVBD in TFSW and hence were discarded. All of the treatment solutions, except the positive control (female prostomial homogenate) are unsuccessful at the induction of GVBD in the prophase I arrested oocytes (Table 5.1).

Arenicola marina

All the treatments, except the positive control (CMF), fail to induce meiotic maturation in the prophase I arrested oocytes of *Arenicola marina* (Table 5.2).

Treatment	Oocyte Activation (mean % GVBD ± SE)
TFSW	0
FPH	99.8 ± 0.2
1% ethanol	2.6 ± 0.4
ionophore A23187	3.6 ± 3.6
arachidonic acid	0.9 ± 0.3

Table 5.1: Mean percentage number of *Arenicola defodiens* oocytes (± standard error of the mean, SE) undergoing GVBD after incubation with different treatments. The results for mean percentage oocyte activation per treatment were pooled for all the concentrations tested..

Female Batch Number	Treatment	Oocyte Activation (mean % GVBD ± SE)
1	TFSW	0.22 ± 0.22
1	CMF	68.4 ± 4.2
1	1% ethanol	2.5 ± 1.0
1	Arachidonic acid	0.1 ± 0.0
2	TFSW	0
2	CMF	73.2 ± 6.7
2	Excess K^+	0
2	Excess Ca^{++}	0
2	Trypsin	0
2	Excess NH_4^+	0
2	FSW, pH6 - pH12	0

Table 5.2: Mean percentage number of *Arenicola marina* oocytes (± standard error of the mean, SE) undergoing GVBD after incubation with different treatments. The results for mean percentage oocyte activation per treatment were pooled for all the concentrations tested.

5.3.2 Activation of Metaphase I Oocytes - *A. marina*

Parthenogenic activation of the oocytes occurred in response to calcium ionophore A23187 above concentrations of 10^{-6}M (Fig. 5.1). Approximately 25% of the oocytes incubated in 1% ethanol (negative control for the 10^{-5}M ionophore solution) also exhibited a raised fertilization envelope (Fig. 5.1). Polar bodies were located on many of the oocytes fertilized by sperm and also those that had undergone parthenogenic activation with calcium ionophore (10^{-6}M or 10^{-5}M). Nevertheless, the number of oocytes with polar bodies was not recorded - accurate counting of these bodies is very difficult, as oocytes usually lie on their flatter side where the polar bodies are situated and obscured from view.

For statistical analysis, the data, from parthenogenic activation by ionophore, were recorded as proportions and arc-sine transformed. To determine if these data were suitable for ANOVA (Analysis of Variance), a

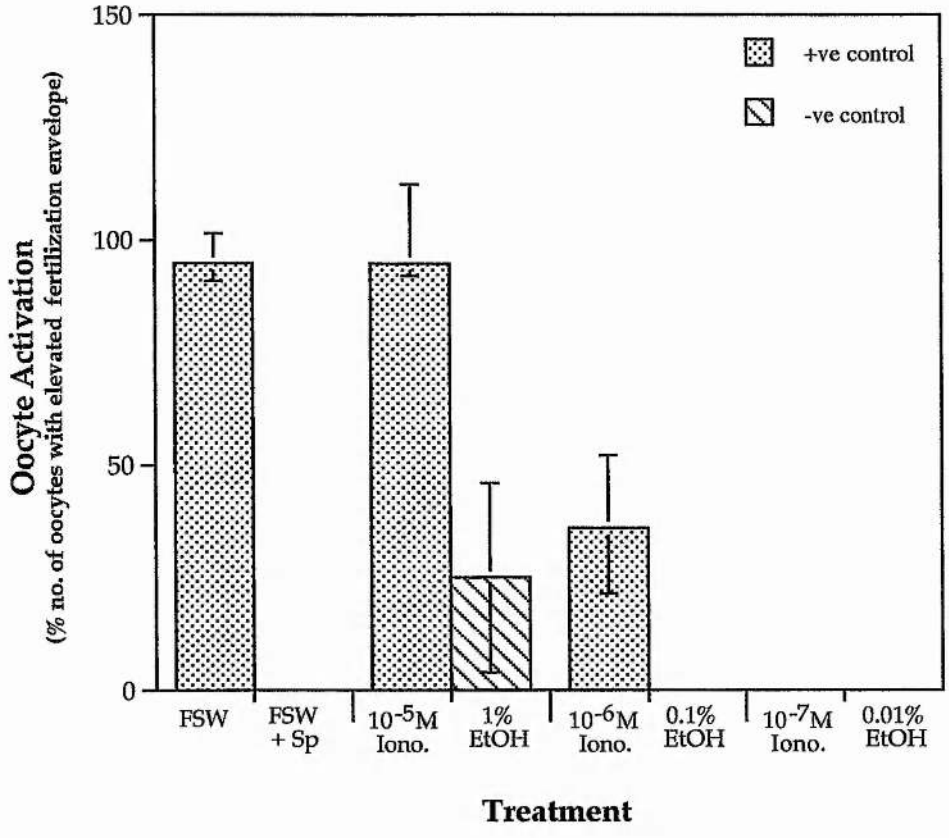


Figure 5.1: Mean percentage parthenogenic activation (assessed by fertilization envelope elevation) of *Arenicola marina* oocytes, with different treatments. The data presented is arc-sine back transformed. Bars = standard error of the mean. Sp = sperm; Iono. = Ionophore A23187; EtOH = Ethanol.

test for homogeneity of variance using a F_{max} test was carried out. The value for F_{max} (282.3) was less than the critical value for $F_{8, 2}$ and therefore the variances are not significantly different and a one-way ANOVA was carried out. The difference in mean oocyte activation between the treatments using a one way ANOVA is statistically significant ($F_{7,16} = 32.8$, $p < 0.01$) and to determine where the differences lay, a Tukey test was carried out. Not all the samples were compared with each other as for each concentration of ionophore treatment there was an individual negative control containing ethanol at the appropriate concentration and hence only relevant to one sample.

Treatment	-ve control for the treatment	Significant Diff. (p , 0.05%)	+ve control for the treatment	Significant Diff. (p < 0.05%)
FSW + Sp	TFSW	Yes	-----	-----
10-5M Iono.	1% EtOH	Yes	TFSW + Sp	No
1% EtOH	TFSW	Yes	-----	-----
10-6M Iono.	0.1% EtOH	Yes	TFSW + Sp	Yes
0.1% EtOH	TFSW	No	-----	-----
10-7M Iono.	TFSW	No	TFSW + Sp	Yes
0.01% EtOH	TFSW	No	-----	-----

Table 5.4: Tukey test summary table for the data collected on parthenogenic activation of *Arenicola marina* oocytes using calcium ionophore A23187. The positive and negative controls are given for each treatment. Sp = sperm; Iono. = Ionophore A23187; EtOH = Ethanol.

Table 5.4 shows that a concentration of 10^{-6}M ionophore A23187 or above results in parthenogenic activation in a significant number of oocytes. There is no difference between the positive control (sperm) and 10^{-5}M ionophore, however oocyte activation at 10^{-6}M ionophore was significantly lower. The percentage oocyte activation at 10^{-5}M ionophore was significantly different from the negative control of 1% ethanol. However, 1%

ethanol itself significantly induced oocyte activation, when compared to TFSW (Table 5.4 and see Fig. 5.1). Hence the activation of the oocytes treated with 10^{-5}M ionophore is not due to ionophore alone but also due to the effects of the 1% ethanol solution. Overall we can state that ionophore can induce parthenogenic oocyte activation at a concentration equal to or greater than 10^{-6}M.

Changing the pH levels of the seawater and excess potassium or calcium ions does not induce parthenogenic activation of *Arenicola marina* metaphase I arrested oocytes (Table 5.5). In addition, incubation in calcium-free seawater resulted in abnormal looking oocytes as the "fertilization envelope" was raised to an exaggerated extent. In many cases, the extent of the fertilization envelope elevation from the oocyte was so great that it "burst". Furthermore, calcium-free seawater induced extensive clumping in the oocytes. Activation in response to calcium-free seawater and calcium-free seawater plus sperm, was therefore not included in the results.

Female Batch Number	Treatment	Oocyte Activation (% no. of oocytes with FE, ± SE)
1	FSW	0
1	FSW + Sp	97.8 ± 0.6
1	Excess K^+ ions	0
1	Excess Ca^{++} ions	0
2	FSW	0
2	FSW + Sp	100
2	pH6 - pH10	0
2	Excess NH_4^+ ions	0.5 ± 0.2

Table 5.5: Mean percentage number of *Arenicola marina* oocytes (± standard error of the mean, SE) undergoing fertilization envelope (FE) elevation after incubation with different treatments. The results for oocyte activation per treatment are pooled for all the concentrations tested.

5.4 DISCUSSION

Incubation in seawater containing excess potassium ions induces a depolarisation of the oocyte plasma membrane and triggers an influx of calcium ions from the external medium. This treatment is unsuccessful in the release of the prophase I block in *Arenicola marina* oocytes, as is incubation in seawater contains excess calcium ions. Furthermore, the use of chemicals that induce an increase in calcium levels from intracellular stores is also ineffective (Watson *et al.*, 1998). These results show collectively that raising intracellular calcium alone, from either internal or external sources, is not sufficient to release the prophase I block in *A marina*. These data strongly support the hypothesis that calcium is not the second messenger in the transduction of the CMF signal to MPF activation. This agrees with the results obtained with other marine invertebrates such as starfish (Kikuyama and Hiramoto, 1991; Stricker *et al.*, 1994) but is in contrast to the prophase I oocytes of other polychaete species investigated to date, which will mature in chemicals that alter intracellular calcium concentration (refer to Table 5.6). Direct evidence, however, that calcium is the native second messenger in these polychaete oocytes is not available. An induced rise in calcium, caused by the chemicals tested, may be activating these oocytes by another pathway different from that used during *in vivo* maturation.

It should be noted that although calcium is clearly not the main signal involved in *Arenicola marina* oocyte maturation, whether or not these cations facilitate the process cannot be confirmed. Further work should be carried out on whether release of the prophase I block in *A. marina* oocytes can occur in absence of calcium. This could be achieved by rinsing the prophase I oocytes in calcium-free seawater, followed by incubation in active CMF that also contained a calcium chelating buffer such as EGTA (ethyleneglycol-bis[β–aminoethyl ether] N,N,N',N'tetraacetic acid) or EDTA

(ethylenediaminetetraacetic acid).

As with all other organisms examined except molluscs (section 1.3.3), pH change does not induce resumption of meiosis in the prophase I arrested oocytes of *Arenicola marina*. Arachidonic acid, and its metabolites (specifically HETE) can induce meiotic reinitiation in the oocytes of starfish (Meijer *et al.*, 1986a) and the surf clam *Spisula solidissima* (Varaksin *et al.*, 1992) but as shown here, this is ineffective in the oocytes of both *A. marina* and *A. defodiens*.

In summary, none of the chemicals tested to date are able to mimic the CMF-induced meiotic maturation in the oocytes of *Arenicola marina* (see Table 5.6). Results indicate, however, that the signal transduction pathway from PMH (prostomial maturation hormone) to activation of MPF in *A. defodiens* oocytes involves calcium (see Table 5.6). This highlights further differences between the oocyte maturation mechanisms of the two *Arenicola* species (see also Watson *et al.*, 1998).

The only treatment that induces parthenogenic activation in the oocytes of *Arenicola marina* is calcium ionophore A23187. This indicates that, as with all other species, calcium is the intracellular ionic effector that triggers oocyte activation after fertilization. In deuterostomes, the release of calcium at fertilization is thought to occur in response to the production of inositol trisphosphate (IP_3) which induces the opening of calcium channels within the endoplasmic reticulum (Berridge, 1993; see Whitaker and Swann, 1993, for review). Insemination or injection of IP_3 into starfish oocytes which have undergone GVBD, at which time fertilization normally occurs (Miyake and Hirai, 1979), results in complete release of the intracellular calcium stores (Chiba *et al.*, 1990). However, prophase I oocytes respond to the same treatments (IP_3 injection and sperm entry) with only a minimal release of

Treatment	*Arenicola marina*	*A. defodiens*	*Chaetopterus pergamentaceus*	*Sabellaria alveolata*
Ca^{++}CHANGES				
Iono. A23187	-	-	+	+
Tetracaine	-	+	+	
Procaine	-	+	-	
Propranolol	-	+		
Oxprenolol	-	+		+
$LaCl_3$	-	+		+
Excess K^+ ions	-		+	
Excess Ca^{++} ions	-		+	
Maturation in Ca-free SW		+		+
H^+ CHANGES				
Increased pH	-			
Decreased pH	-			
Excess NH_4^+	-			
OTHERS				
DTT	-	+		
Arachidonic acid	-	-		
Trypsin	-		+	+
Serotonin	-			
References	Watson (1996); Watson et al (1998c)*	Watson *et al* (1998c); Meijer (1980)	Ikegami *et al* (1976); Eckberg and Carrol (1974)	(Peaucellier, 1977)

* results from this chapter are published in Watson *et al* (1998).

Table 5.6. Showing the action of different chemicals in the induction of the prophase I to metaphase I transition in polychaete oocytes. + = GVBD; - = no GVBD.

calcium even though the intracellular stores of these cations are present and already established (Chiba *et al.*, 1990). Unlike starfish, the oocytes of *Arenicola marine* and *A. defodiens* show no activation response to spermatozoa at the prophase I stage. The "unfertilizability" of these oocytes could be due to the mechanism that releases the calcium stores, being inert in prophase I oocytes and only becoming established during oocyte maturation. This is supported by the fact that both *A. marina* and *A. defodiens* prophase I oocytes do not respond to ionophore A23187 and yet the metaphase I oocytes do (this study; Meijer, 1979b; Meijer, 1980; Watson *et al.*, 1998). Furthermore, an investigation by Meijer (1979b) shows that fertilization by spermatozoa or parthenogenic activation by ionophore A23187 is only possible after GVBD in *A. defodiens* oocytes. It, therefore, appears that as with starfish oocytes, the calcium-sensitive release mechanism is not receptive in prophase I oocytes and only become functional during maturation. Overall, this evidence suggests that the acquisition of fertilizability may be tied to the calcium-release mechanisms becoming established during the prophase to metaphase I transition.

Jaffe (1991) proposed that because protostomes can be parthenogenically activated by surplus potassium ions (as opposed to deuterostomes) then the intracellular calcium increases recorded at fertilization must occur solely by an influx from the external medium. If this were true one would expect excess potassium ions to induce parthenogenic activation in all protostome oocytes and for fertilization in calcium-free seawater to be impossible. In support of Jaffe's hypothesis, in the oocytes of protostome species which are fertilized at prophase I, activation cannot occur in the absence of calcium (see Table 5.7). However, all the mollusc species that belong to class II (i.e. oocytes which are fertilized at metaphase I) can be activated in calcium free seawater (Table 5.7). In addition, the metaphase I oocytes of *Arenicola marina* cannot be activated by excess potassium ions.

Hence from the results collected so far, it is apparent that Jaffe's hypothesis should be redefined. The evidence collectively agrees with the proposal first stated by Deguchi and Osanai (1994b) whereby all protostome oocytes that are fertilized at prophase I require an external source of calcium ions for activation. However, whether protostome oocytes fertilized at metaphase I require calcium in the external medium, is dependent upon the species (see Table 5.7).

	Polychaetes					Molluscs									Ech*	Ne**
Species	*NEREIS*	A. MARINA	A. DEFODIENS	CHAETOPERUS	PECTINARIA	*BARNEA*	*SPISULA*	*MACTRA*	HIATELLE	LIMARIA	CROSSOTREA	RUDITAPES	PATELLA	MYTILUS	*URECHIS*	CEREBRATULUS
Stage at Fertilization	*P*	M				*P*			M						*P*	M
Ionophore A23187		+	+		+		+	+				+	+	+		
Excess K^+ ions	+	-		+		+	+	+				+	+	+		+
External Ca^{++} required ?***					+	+	+	+	-	-	-		-	-	+	+
References ****	1.	2.	3.	4.	5.	6.	7,8.	9.	10.	10.	10.	11.	12.	10, 11, 13	14.	15.

* Echiuroid
**Nemertean
***with ionophore A23187 or sperm
****References: 1. (Heilbrunn & Wilbur, 1937) 2. Watson *et al.*, (1998c); 3. (Meijer, 1979b); 4. Eckberg and Miller (1995); 5. Anstrom and Summers (81); 6. [[Dubé, 1982 #574] 7. Schuetz, 1975 #346], 8. (Dubé, 1988); 9. (Deguchi and Osanai, 1994b) 10. (Deguchi & Osanai, 1994a) 11. (Abdelmajid *et al.*, 1993b); 12. (Guerrier *et al.*, 1986) 13. (Dufresne-Dubé *et al.*, 1983a); 14. (Stephano and Gould, 1997); 15. (Stricker, 1996)

Table 5.7. Showing the action of ionophore A23187, excess K+ ions, and external calcium ions on the activation of protostome oocytes. P = prophase I of meiosis; M = metaphase I of meiosis.

CHAPTER 6

REGULATION OF M-PHASE PROMOTING FACTOR (MPF) DURING MEIOTIC MATURATION IN *NEREIS VIRENS* OOCYTES

6.1 INTRODUCTION

Activation of the M-phase promoting factor (MPF) leads to meiotic and mitotic entry. To activate MPF, the two subunits cdk1 and cyclin B must become associated (see section 1.5.1). In most organisms, cyclin B is synthesised during interphase and recruits the already present cdk1 subunits (Meijer *et al.*, 1991). Once a complex is formed, two inhibitory residues (threonine 14 and tyrosine 15) become phosphorylated to prevent premature activation of MPF until the cell is ready to enter M-phase (see section 1.5.1). At activation, first threonine 14 and then tyrosine 15 become dephosphorylated (Borgne and Meijer, 1996). This creates a highly transient intermediate form of tyrosine-only phosphorylated cdk1, before full dephosphorylation and activation occurs (Borgne and Meijer, 1996). A third residue is also important in the activation of MPF, threonine 161, and in contrast to threonine 14 and tyrosine 15, this residue must be phosphorylated (Ducommun *et al.*, 1991; Fesquet *et al.*, 1993).

Oocyte maturation is an excellent cellular mechanism with which to study the regulation and activation of MPF. Hence MPF has been studied during meiotic reinitiation in the oocytes of a wide range of organisms including echinoderms; molluscs; ascidians; fish; amphibians and mammals (see for examples Arion *et al.*, 1988; Labbé *et al.*, 1989a; Abdelmajid *et al.*, 1994; Verlhac *et al.*, 1994; Tanaka and Yamashita, 1995). MPF regulation, however, has been investigated to date in only one species of polychaete, *Chaeopterus pergamentaceus* (Eckberg *et al.*, 1996). Interestingly, MPF regulation in this polychaete exhibits a different mechanism of activation. As with other organisms, prophase I oocytes of *C. pergamentaceus* contain a latent inactive form of cdk1 complexed to cyclin B, pre-MPF (Eckberg *et al.*, 1996; Eckberg, 1997). In contrast, however, cdk1 is not tyrosine phosphorylated in prophase I oocytes, no dephosphorylation occurs during

activation and hence the precursor form is not maintained inactive by inhibitory phosphorylations (Eckberg *et al.*, 1996; Eckberg, 1997). Eckberg (1997) hypothesised that MPF activation is triggered in these oocytes by phosphorylation of the threonine 161 residue, by protein kinase C.

It is important to investigate the activation of MPF in different species and phyla to discover the features common to all eukaryotic organisms and to highlight the differences. Together the information should help us move towards a greater understanding of the regulation of this enzyme. The aim of the studies reported in this chapter is to describe the regulation of MPF activity, particularly of the cdk1 subunit, during oocyte maturation in the polychaete *Nereis virens*.

6.2 Materials and Methods

6.2.1 Solutions

Antibodies

Monoclonal anti-PSTAIRE antibodies were raised against the peptide sequence NH_2-EGCPSTAIRESLLKEGGC-COOH (donated by Dr M. Yamashita); polyclonal anti-cyclin B (cyclin B was derived from the oocytes of the amphibian *Bufo bufo* and raised in rabbit) (donated by Dr T. Kishimoto); polyclonal phosphotyrosine antibodies (donated by Dr J.Y.J. Wang); polyclonal anti-GEGTYG antibodies were raised against the peptide sequence NH_2-VEKIGEGTYGVVVKARHKLS-COOH (donated by Dr H.Y.L. Tung).

Buffers

Bead Buffer: 50mM Tris-HCl, pH 7.4, 5mM NaF, 250mM NaCl, 5mM EDTA, 0.1% Nonidet P-40, 5mM EGTA, 10 $\mu g.ml^{-1}$ leupeptin, 10 $\mu g.ml^{-1}$ aprotinin, 10 $\mu g.ml^{-1}$ soybean trypsin inhibitor, and 100mM benzamidine.

Buffer C: 25mM MOPS, pH 7.0, 60mM β-glycerophosphate, 30 mM *p*-nitrophenylphosphate, 5mM EGTA, 15mM $MgCl_2$, 1mM DTT and 0.1mM sodium orthovanadate.

Homogenisation Buffer: 25mM MOPS, pH 7.2, 60mM β-glycerophosophate, 15mM *p*-nitrophenylphosphate, , 15mM EGTA, 15mM $MgCl_2$, 2 mM DTT, 1mM sodium orthovanadate, 1mM sodium fluoride, 1mM disodium phenylphosphate, 10 $\mu g.ml^{-1}$ leupeptin, 10 $\mu g.ml^{-1}$ aprotinin, 10 $\mu g.ml^{-1}$ soybean trypsin inhibitor, and 100mM benzamidine.

Sodium Bicarbonate-buffered saline (SBBS): pH 8.2, 200 mM sodium carbonate and 200 mM sodium chloride.

Buffers used for semi-dry blotting: Anode buffer 1 (0.3M Tris, 20% methanol, pH 10.4); Anode buffer 2 (0.025M Tris, 20% methanol, pH 10.4). cathode buffer (0.025M Tris, 0.04M glycine, 20% methanol, pH 9.4).

Tris-Buffer A (TBA): 50mM Tris, pH 8, 50mM NaCl, 1mM EDTA and 1mM DTT.

Tris-Buffered Saline (TBS): 50mM Tris, pH 7.4, 150mM NaCl, and 0.1% Tween 20.

6.2.2 Methodology

This work utilises affinity chromatography using $p9^{CKShs1}$-coated sepharose beads to separate the cdk1/cyclin B from the other cell constituents. This protein has been shown to selectively bind to cdk1/cyclin B in the cells of organisms from starfish to humans (Borgne and Meijer, 1996). cdk1 selectively binds to the $p9^{CKShs1}$-sepharose beads, and cyclin B will also co-elute when using this technique, if bound to cdk1. cdk1/cyclin B regulation in starfish oocytes is an extremely well-studied system and has been studied extensively (see Meijer and Mordret, 1994). For this reason cdk1/cyclin B from starfish oocytes will be used as a positive control throughout these experiments, in parallel with the cdk1/cyclin B from polychaete oocytes.

The phosphorylation state of the cdk1 subunit was identified by a combination of 2 methods:

Observation of Changes in Electrophoretic Mobility.

Phosphorylated cdk1 is the slowest migrating form and is observed as an "upper form" (Borgne and Meijer, 1996). Dephosphorylation causes the protein to migrate faster and hence is observed as a "lower form" (for examples see Norbury *et al.*, 1991; Borgne and Meijer, 1996).

Cross-reactivity with Various Antibodies.:

The anti-PSTAIRE antibody was directed against a peptide containing a specific sequence that is conserved in cdk1. anti-PSTAIRE will bind to all forms of cdk1, irrespective of the phosphorylation state: threonine (T) and tyrosine (Y) phosphorylated (p) cdk1 ("Tp-Yp"), the intermediate form with a phosphotyrosine only "T-Yp", and the unphosphorylated form "T-Y". The anti-phosphotyrosine antibody will only bind to cdk1 containing a phosphotyrosine residue, "Tp-Yp" and "T-Yp". Finally, the anti-GEGTYG antibody was raised against a peptide sequence of cdk1 containing the threonine 14 and tyrosine 15 residues (dephosphorylated) and will cross-react with dephosphorylated active cdk1, "T-Y". However, as demonstrated by Borgne and Meijer (1996) this antibody will also bind with the intermediate form "T-Yp" in the oocytes of starfish.

6.2.3 Preparation of the Oocytes

Nereis virens

The gametes to be used during these studies were first assessed for their viability to fertilize (see section 2.2.2). Prophase I oocytes were then withdrawn from the coelomic cavity of several female *Nereis virens* (for details refer to section 2.2.2). The oocytes were washed in twice filtered seawater (TFSW) and 50 µl samples were placed in Eppendorf microtubes. About a quarter of the samples were frozen immediately in liquid nitrogen

(N_2). The remaining prophase I oocytes were fertilized by the addition of active spermatozoa (withdrawn from males using a hypodermic syringe). Fertilized oocyte samples were then frozen in liquid N_2 at fixed time intervals (every 10 or 20 minutes) for up to 150 minutes. All the oocytes were stored, until ready for use, in a liquid N_2 biostore or a -70 or -80 °C freezer.

Starfish

The gonads were removed from specimens of *Marthasterias glacialis*, and gently prised apart in calcium-free seawater (this prevents spontaneous maturation) to release the oocytes. The oocytes were collected and washed 3 times in calcium-free seawater. 1 ml of oocytes was taken and fifty microlitres aliqouts were placed in Eppendorf tubules and then frozen in liquid N_2. The remaining oocytes were placed into a solution of 10 µm 1-Methyladenine (in natural seawater) to induce maturation. The oocytes were examined under light microscopy at regular intervals and following GVBD, 50 µl aliquots were placed into Eppendorf tubules and frozen in liquid N_2.

6.2.4 Determination of GVBD - *Nereis virens*

The oocytes of *Nereis virens* contain large green-coloured yolk granules, which obscures observation of the germinal vesicle. In addition, following fertilization a large extracellular jelly coat is produced, enhancing the difficulty in observation. Therefore, during fertilization, in addition to being frozen, some oocytes were fixed for light microscopy at each time interval, as described in section 2.2.3. The fixed oocytes were embedded in LR white resin and semi-thin sections were cut and stained (see section 2.2.3). These were observed by light microscopy and the oocytes at each post-fertilization stage were scored for GVBD.

6.2.5 Purification of MPF

cdk1/cyclin B was separated from the oocytes by affinity chromatography onto $p9^{CKShs1}$ coated sepharose beads. The production of $p9^{CKShs1}$-coated speharose beads is documented by Azzi *et al.*, 1994, a description of which is provided below.

$p9^{CKShs1}$ coated sepharose beads

$p9^{CKS}$ is a protein which has a strong and selective affinity for cdk1 with which it forms a very stable association (see Azzi *et al.*, 1994). $p9^{CKShs1}$ was purified from an over-producing strain of *Escheria coli.* The bacterial extract was diluted in distilled water (final protein concentration = 1 mg/ml.) and mixed with a S-sepharose bead solution. The beads were then packed in a column and washed with 20 mM sodium bicarbonate and eluted with sodium bicarbonate-buffered saline (SBBS). Further purification was carried out on a 100 x 2.6 cm Sephacryl S-200 column. Nine-milliltre fractions were collected at a flow-rate of 1ml/min. The elution pattern was monitored by SDS-PAGE and coomassie blue staining or immunoblotting using anti-$p9^{CKShs}$ antibodies. $p9^{CKShs1}$ was conjugated to CNBr-activated Sepharose 4B. Unreacted groups on the resin were quenched with 1 M ethanolamine, pH 8.0. The concentration of coupled proteins per milligram of cell was 3.9 mg for $p9^{CKShs1}$. Just before use, 10 µl of packed protein beads were washed in 1ml of bead buffer and resuspended in 400 µl of bead buffer.

Separation of cdk1/cyclin B

This entire method was carried out in an Eppendorf tubule. Two hundred microlitres of homogenisation buffer was added to each 50 µl oocyte sample and homogenised by ultrasound for 10 - 20 seconds, using a titanium probe, and placed onto ice. The oocyte homogenates were

centrifuged at 3000 x g at 4°C for 10 minutes. The supernatant from each sample was removed and added to 10 µl of washed $p9^{CKShs1}$ beads in 400 µl of bead buffer before placing onto a rotator at 4°C for 30 minutes. The beads were then washed three times in ice cold bead buffer. To recover the proteins from the beads, the final bead buffer wash was removed and 50 µl of Laemmli sample buffer was added to each sample and placed on a hot-plate (90 - 100 °C) for 3 minutes.

6.2.6 Gel Electrophoresis and Western Blotting

Throughout this procedure, cdk1/cyclin B from starfish oocytes (positive control) was run alongside cdk1/cyclin B separated from *Nereis virens*. In addition, the antibodies were tested against the peptide they were raised against, to confirm binding.

The recovered protein samples (cdk1/cyclin B from the oocytes of starfish and *Nereis virens*) were run on 0.75mm thick 10% SDS polyacrylamide gels: seperating gel (1 gel = 5.84ml Acrylamide/bis, 4.38ml 1.5M Tris pH 8.8, 6.89 ml of Milli-Q water, 350 µl of 10% SDS, 30 µl of ammonium persulfate, 30 µl of TEMED); stacking gel (1 gel = 1.25 acrylamide/Bis, 1.86 0.5M Tris pH 6.8, 4.28 ml of Milli-Q water, 75 µl 10% SDS, 100 µl of 10% ammonium persulfate, 50 µl of TEMED). Low-range molecular weight pre-stained SDS-PAGE standards (BioRad) were used for markers.

For Western blotting, the proteins from the gels were transferred to 0.1 µm Protran ® BA-S reinforced nitrocellulose membranes (Schleicher and Schuell) using a semi-dry technique on a Millipore graphite electroblotter system. The gel was placed onto the nitrocellulose membrane (previously equilibrated in cathode buffer). Three sheets of filter paper (Whatmann 3mm) were soaked in cathode buffer and placed on top of the gel. Three

sheets of filter paper were soaked in anode buffer (one In anode buffer 2 and two in andoe buffer 1). The sandwhich was placed in the electropblotter. Transfer occurred over a 30 minute period at 1.5 mA per cm^2. Following protein transfer, the nitrocellulose sheet was cut into two and this separated the heavier cyclin B subunits from the lighter cdk1 subunit. The membranes were placed into a 5% powdered milk (fat free) solution made up in Tris-buffered saline (TBS) and left under constant agitation for one hour. After rinsing in TBS, the membranes were placed into the appropriate primary antibody solution for one hour: anti-PSTAIRE (1:2000); anti-phosphotyrosine (1:1000); anti-GEGTYG (1:1000); anti-Cyclin B (1:1000). This was followed by rinsing in TBS (x3) and then into the appropriate solution of horseradish peroxidase tagged secondary antibodies (1:1000): for anti-GEGTYG antibodies, anti-rabbit secondary antibodies were used and for all other primary antibodies, anti-mouse secondary antibodies were used. All antibody solutions were made up in TBS buffer. Following rinsing of the membranes, the Western blots were analysed by chemoluminescence using ECL reagents and developed into photographs using Hyperfilm, HP.

6.2.7 Histone H1 Kinase Assay

Complexes of cdk1/cyclin B were purified by affinity chromatography onto $p9^{CKShs1}$ coated sepharose beads, as described above. The final bead buffer wash was removed and replaced with 10 µl of buffer C and 5 µl of histone H1 ($5mg.ml^{-1}$), per Eppendorf microtube containing 10 µl of cdk1/cyclin B-coated beads extracted from 50 µl oocyte samples. To this mixture, 5 µl of 90µM [γ-^{32}P]ATP was added and the Eppendorf microtubes were placed on ice to prevent the reaction from starting. Each sample was mixed briefly and then placed into a heated water bath at 30°C for a total of 10 minutes, throughout which time the samples were mixed every 2 minutes. The samples were then placed on ice to stop the reaction. After a brief

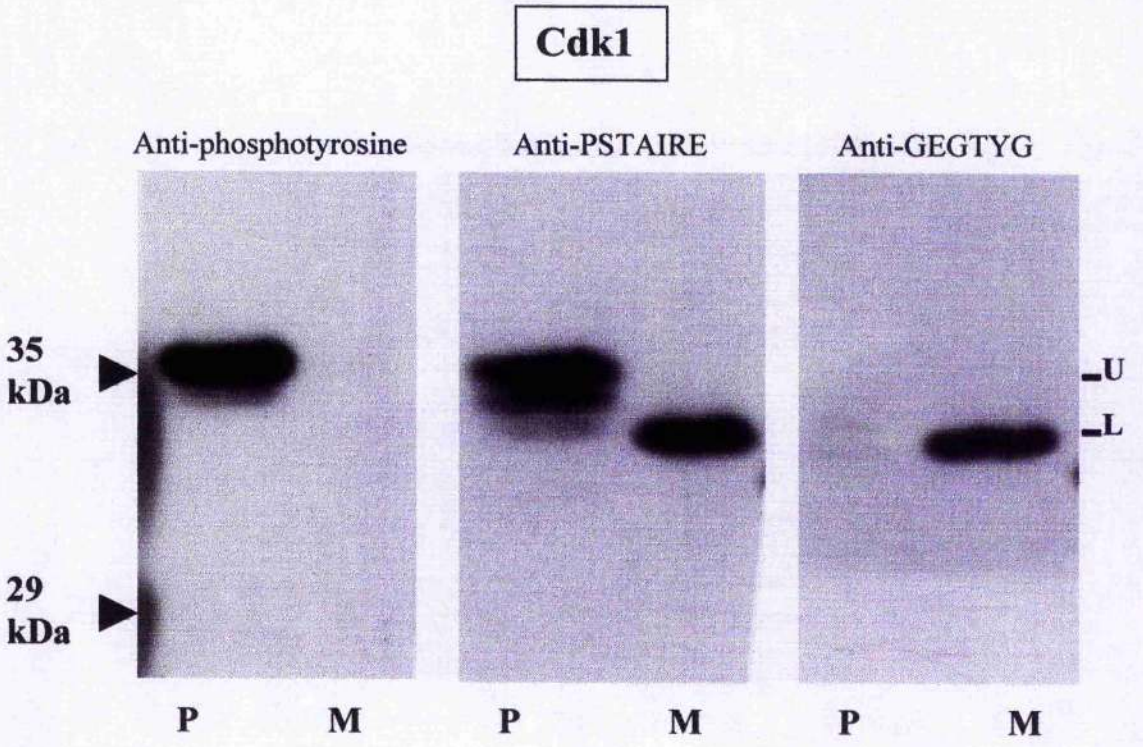
centrifugation, 20 µl of the liquid was removed from each Eppendorf microtuble and placed onto a piece of Whatman p81 phosphocellulose paper. The papers were washed 3 times for 15 - 20 minutes each in 1% phosphoric acid. The papers were removed and placed on tissue paper to dry and then placed into scintillation vials with the addition of 1.5 ml of scintillation fluid. The radioactivity ([^{32}P]phosphate incorporation into histone H1) was measured using a Packard scintillation counter.

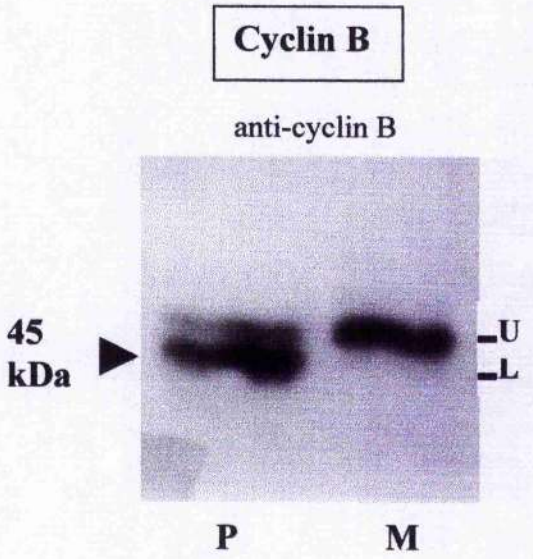
6.3 RESULTS

6.3.1 Controls

For the positive control the anti-PSTAIRE and anti-GEGTYG antibodies were cross-reacted with the peptide they were made against to confirm binding (data not shown). In addition, cdk1/cyclin B separated from starfish oocytes was run in the gels and blotted, alongside the cdk1/cyclin B separated from the *Nereis virens* oocytes. Figure 6.1 shows the Western blots of cdk1/cyclin B purified from the starfish oocytes. The regulation of cdk1/cyclin B in starfish has been studied previously and is used in these methods purely as a positive control. However, in prophase I oocytes all the cdk1 is bound to cyclin B. cdk1 is phosphorylated at tyrosine 15 and threonine 14 and becomes dephosphorylated during the prophase I to metaphase I transition (Borgne and Meijer, 1996). The blots show that the antibodies used were cross-reacting with cdk1 and to the appropriate forms of cdk1 (Fig. 6.1) i.e. the anti-PSTAIRE antibody cross-reacted all forms of cdk1, irrespective of its phosphorylation state (Fig. 6.1A); anti-phosphotyrosine antibody cross-reacted with phosohrylated cdk1 only (separated from the prophase I oocytes, Fig. 6.1B) and the anti-GEGTYG antibody cross-reacted with the unphosphorytaed forms of cdk1 (separated

Figure 6.1. Positive controls. Western blots of the MPF subunits from prophase I (P) and metaphase I (M) oocytes of starfish. cdk1 cross-reacted with anti-PSTAIRE; anti-phosphotyrosine, anti-GEGTYG and anti-cyclin B. U, upper form; L, lower form.


Cdk1
Anti-phosphotyrosine
Anti-PSTAIRE
Anti-GEGTYG
35
kDa
29
kDa
U
L
P
M
P
M
P
M


Cyclin B
anti-cyclin B
45
kDa
U
L
P
M

from the metaphase I oocytes, Fig. 6.1C). Furthermore, the anti-cyclin B antibodies cross-reacted with the cyclin B from the starfish oocytes (Fig. 6.1D).

6.3.2 Activation of cdk1/Cyclin B

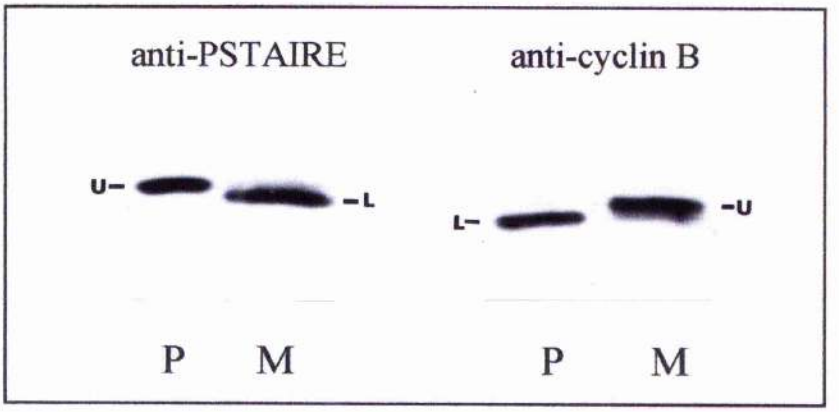
In the prophase oocytes of *Nereis virens*, only one migrating form of cdk1 is present, the upper form (Fig. 6.2A). During the prophase to metaphase transition, the cdk1 protein undergoes a downward shift creating the lower form, indicating that dephosphorylation had occured (Fig. 6.2A). In contrast to the cdk1 subunit, cyclin B is converted to a slower migrating form, indicating that phosphorylation of this subunit has occurred (Fig. 6.2A).

Fig 6.2B shows the kinase activity of cdk1/cyclin B in prophase I and metaphase I oocytes. Observation of the figure reveals that in association with the changing electrophoretic mobility of the two subunits, meiotic maturation occurs in conjunction with the activation of MPF. Statistical analysis was performed on the kinase activity data using an unmatched pairs t-test. The calculated value of t (7.47) exceeded the tabulated value of 3.169 and hence the difference between the cdk1/cyclin B activity in prophase and metaphase I oocytes is significant at the 1% level.

The apparent dephosphorylation that occurs in cdk1 during meiotic maturation (Fig. 6.3A) is confirmed by cross-reacting with the anti-phosphotyrosine antibodies (Fig. 6.3B). The cdk1 protein in prophase oocytes is phosphorylated at its tyrosine residue as demonstrated by its excellent cross-reactivity with the anti-phosphotyrosine antibodies (Fig. 6.3B). During meiotic maturation, cdk1 becomes dephosphorylated as shown by the vast reduction in signal of tyrosine-phosphorylated cdk1 at metaphase (Fig. 6.3B). In prophase I oocytes, cdk1 (the upper form) also

Figure 6.2: A) Western blots of the MPF subunits from prophase I (P) and metaphase I (M) oocytes of *Nereis virens*. cdk1, cross-reacted with anti-PSTAIRE; cyclin B, cross-reacted with the anti-cyclin B. U, upper form, L, lower form. B) Mean kinase activity of cdk1/cyclin B (measured by the amount of radioactive phosphate incorporated into histone H1) purified from *Nereis virens* prophase and metaphase I oocytes (vertical bars show standard error of the mean).

A


anti-PSTAIRE
anti-cyclin B
U–
–L
L–
–U
P
M
P
M

B

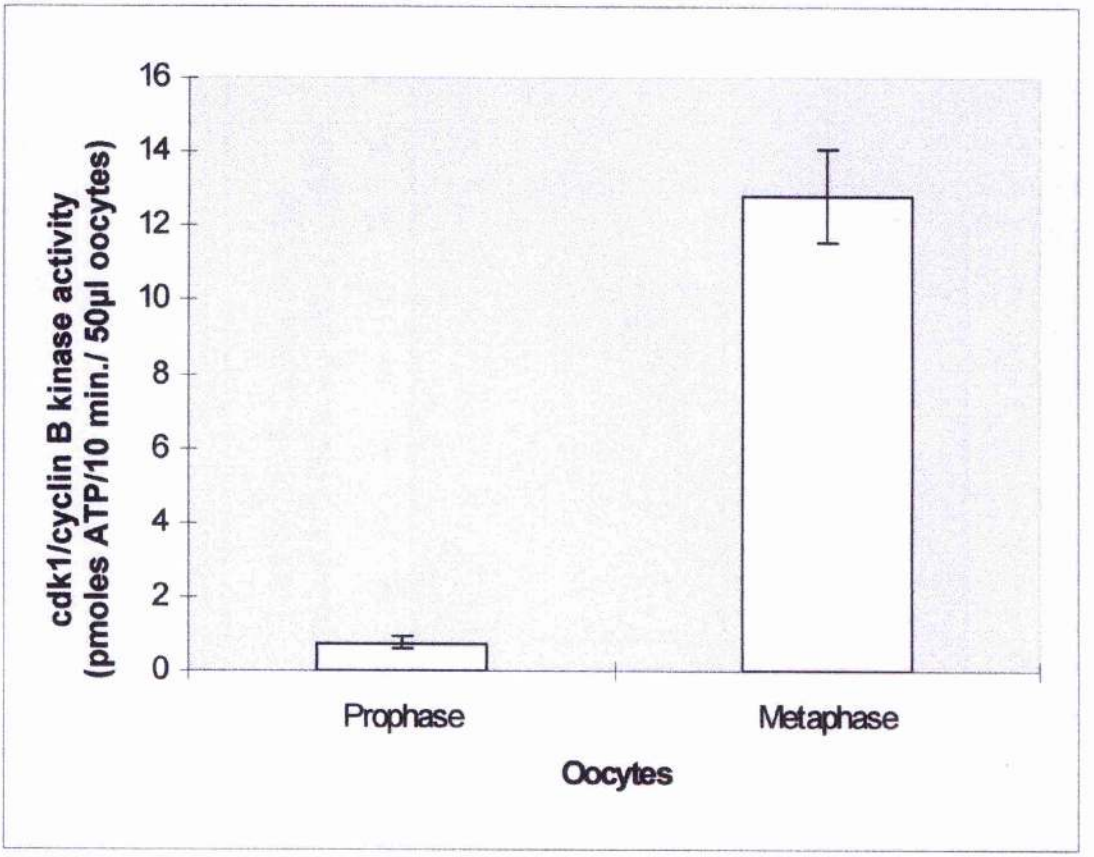
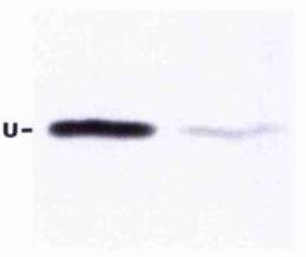

cdk1/cyclin B kinase activity
(pmoles ATP/10 min./ 50µl oocytes)
16
14
12
10
8
6
4
2
0
Prophase
Metaphase
Oocytes

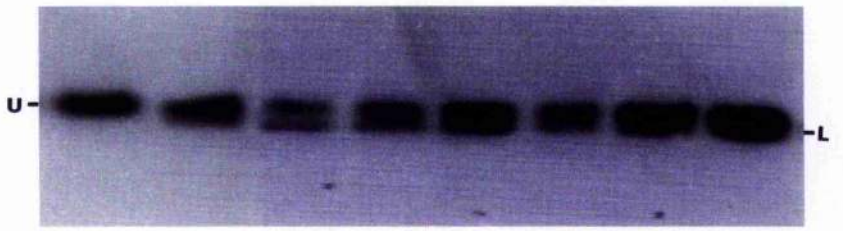
Figure 6.3: Western blots of the cdk1 subunit during the prophase to metaphase I transition, cross-reacted with A) anti-PSTAIRE (same figure as shown in 6.2A), B) anti-phosphotyrosine, C) anti-GEGTYG (0 minutes post-fertilization = prophase I oocyte; 140 minutes post-fertilization = metaphase I oocyte). U, upper form; L, lower form.

A anti-PSTAIRE

B anti-phosphotyrosine



C anti-GEGTYG



0 20 40 60 80 100 120 140

minutes post fertilization

shows a strong signal when cross-reacted with anti-GEGTYG antibodies (Fig. 6.3C). In addition, following fertilization the lower form (indicating dephosphorylation has occurred) is also detected by these antibodies and this band increases in density as post-fertilization time increases (Fig. 6.3C).

6.4 Discussion

As has been observed in other organisms (Pondaven *et al.*, 1990; Gautier and Maller, 1991; Meijer *et al.*, 1991), entry into M-phase in the oocytes of *Nereis virens* is accompanied by the dephosphorylation of cdk1 and the reciprocal phosphorylation of cyclin B. In addition, these changes occur in conjunction with an increase of histone H1 kinase activity, demonstrating that MPF was activated. Again, this is in agreement with the findings of many other authors who have investigated MPF (Dunphy and Newport, 1989; Gautier *et al.*, 1989; Pondaven *et al.*, 1990; Choi *et al.*, 1991; Meijer *et al.*, 1991; Gavin *et al.*, 1994). However, as discussed below, the regulation of the cdk1 subunit during meiotic reinitiation in *N. virens* oocytes also shows significant differences when compared to other organisms.

In other higher eukaryotes, the precursor of MPF is maintained inactive by the phosphorylation of both threonine 14 and tyrosine 15 on the cdk1 subunit (Krek and Nigg, 1991; Norbury *et al.*, 1991). The results presented here, however, indicate that pre-MPF in *Nereis virens* is composed of tyrosine-only phosphorylated cdk1. The reasons for this are as follows: the pre-MPF complex in the prophase I oocytes contains one migrating form of cdk1 (the upper form). This upper form is recognised by all three sets of antibodies (anti-PSTAIRE, anti-phosphotyrosine and anti-GEGTYG). The only form of cdk1 to be recognised by all sets of antibodies is the tyrosine-phosphorylated intermediate form, T-Yp (Borgne and Meijer, 1996). Hence

from the results it appears that cdk1 in *Nereis* prophase I oocytes is in the form T-Yp (see Fig. 6.4). These results indicate that dephosphorylation of tyrosine only on the cdk1 subunit leads to MPF activation and meioitc reinitiation.

The only organism that is known to contain pre-MPF with a tyrosine phosphorylation only (i.e. without a threonine 14 phosphorylation) is that of the fission yeast *Schizosaccharomyces pombe* (Gould and Nurse, 1989) and the budding yeast *Saccharomyces cerevisiae* (Amon *et al.*, 1992). In the former, the cdk1 subunit is phosphorylated at tyrosine 15 in pre-MPF while in the latter an equivalent residue (tyrosine 19) is phosphorylated. So as with yeast, it appears that in the oocytes of *Nereis virens,* threonine 14 (or the equivalent residue) has no function in the inhibition/ regulation mechanism of cdk1. Furthermore, the need for dual inhibitory residues is apparently not necessary. Studies have show that in cdk1, which is normally phosphorylated at the two residues in its inactive form, only one residue needs to be phosphorylated to prevent activation (Norbury *et al.*, 1991). Norbury *et al.* (1991) demonstrated that if tyrosine 15 was replaced with phenylalanine or threonine 14 was replaced with alanine (i.e. preventing phosphorylation at the substituted residues) little effect was observed on the activity of the enzyme. This raises the question is threonine 14 purely a supplemental phosphorylated residue to provide an extra degree of regulatory inhibition?

The dual inhibitory phosphorylations of cdk1 is common within the animal kingdom (e.g. *Xenopus,* starfish, humans) and the activating phosphatase has been characterised. cdc25 is a dual specificity protein phosphatase which dephosphorylates cdk1 and activates MPF (Strausfeld *et al.*, 1991; Jessus and Beach, 1992; see Millar and Russell, 1992, for review). This model, however, is not ubiquitous throughout the eukaryotic

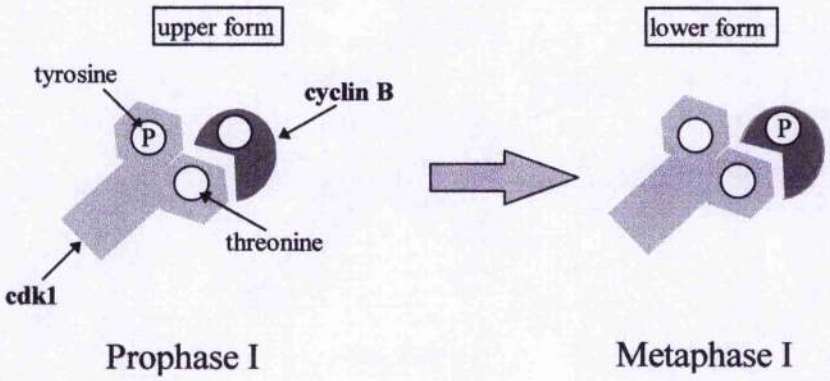


Figure 6.4. Diagrammatic summary of MPF regulation and activation in *Nereis virens* oocytes during meiotic maturation. The prophase oocytes contain the upper migrating form of cdk1 (as observed on Western blots see Fig. 6.3A) which is phosphorylated upon its tyrosine residue (Fig. 6.3B) and bound to cyclin B (Fig. 6.2A). At activation, the tyrosine phosphorylated cdk1 becomes dephosphorylated and is converted to a faster moving form (Fig. 6.3). In addition, the results indicate that there is no secondary threonine inhibitory residue and that the cyclin B subunit becomes phosphorylated (Fig. 6.2 and 6.3).

organisms. In the prophase oocytes of the polychaete *Chaetopterus pergamentaceus*, cdk1 is bound to cyclin B but is not tyrosine phosphorylated and no dephosphorylation occurs during activation (Eckberg *et al.*, 1996). Furthermore, inhibitory phosphorylations upon the cdk1 subunit are not required in fish or amphibian oocytes (except *Xenopus*) as cdk1 is monomeric and cyclin B is the regulator for MPF activation (Yamashita *et al.*, 1992; Katsu *et al.*, 1993; Tanaka and Yamashita, 1995; Kondo *et al.*, 1997; Ihara *et al.*, 1998; Sakamoto *et al.*, 1998).

Overall, active MPF is identical when comparing different species whereby cdk1 is complexed to cyclin B and dephosphorylated upon its tyrosine 15 and threonine 14 residues. In addition threonine 161 (or equivalent) is phosphorylated during activation (although this was not investigated in this study). The precise mechanism of the maintenance of cdk1 in its inactive form and its activation, however, varies between species and is not phylum-specific.

CHAPTER 7

REGULATION AND INHIBITION OF M-PHASE PROMOTING FACTOR (MPF) DURING MEIOTIC MATURATION IN *ARENICOLA MARINA* OOCYTES

7.1 INTRODUCTION

In all organisms, prior to M-phase (i.e. during G_2) cdk1 is present within the cell but maintained in one of two inactive forms: cdk1 is phosphorylated and bound to cyclin B and activated by dephosphorylation (Gould and Nurse, 1989; Krek and Nigg, 1991; Norbury *et al.*, 1991; Amon *et al.*, 1992; Borgne and Meijer, 1996) or cdk1 is unphosphorylated and monomeric and cyclin B is the activating factor (Hirai *et al.*, 1992; Katsu *et al.*, 1993; Yamashita *et al.*, 1995; Haider and Balamurugan, 1996; Kondo *et al.*, 1997; Tanaka and Yamashita, 1995; Sakamoto *et al.*, 1998; Naito *et al.*, 1995).

In cells where pre-MPF is present at G_2, it is activated by cdc25. cdc25 is a dual specificity phosphatase and removes the inhibitory phosphates from both tyrosine 15 and threonine 14 of cdk1 in the pre-MPF complexes, creating the fully active enzyme (Strausfeld *et al.*, 1991; Jessus and Beach, 1992; Honda *et al.*, 1993). Another protein phosphatase, pyp3, has been isolated from yeast (Millar and Russell, 1992) and removes the phosphate group from tyrosine 15 only (Borgne and Meijer, 1996). These activating kinases work antagonistically against the wee1 kinase, the enzyme responsible in part for phosphorylating the cdk1 subunit and preventing activation (Featherstone and Russell, 1991; Parker *et al.*, 1992; McGowan and Russell, 1993). Other enzymes that have been characterised and shown to have a role in the inhibitory phosphorylation of cdk1 are myt1 (Mueller *et al.*, 1995; Liu *et al.*, 1997) and mik1 (Lundgren *et al.*, 1991).

The phosphorylation of MPF at threonine 161 (threonine 167 in yeast) is also a pre-requisite for activation (Fesquet *et al.*, 1993). Phosphorylation of this residue is required in both models of MPF activation (post-translational dephosphorylation or cyclin B synthesis). Threonine 161/167 is phosphorylated by CAK (cdk activating kinase) and the kinase subunit is

encoded by the gene MO15 (Fesquet *et al.*, 1993).

The knowledge of cell cycle regulation is of particular interest to cancer researchers. Evidence shows that mammalian cells that are cancerous are equipped with a malfunctioning cdk regulatory network (Motokura and Arnold, 1993; Peters, 1994). This has lead to a search for inhibitors of cdk's which could also act as tumour suppressers (Rialet and Meijer, 1991; Meijer, 1995; Meijer, 1996). Two such chemical inhibitors discovered are the purine analogues olomoucine (2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine) and roscovitine (2-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine). These chemicals exhibit potent and selected inhibition of certain cdk's and studies reveal that these chemicals prevent cdk1/cyclin B kinase activity (Vesely *et al.*, 1994; De Azevedo *et al.*, 1996).

The regulation and inhibition of MPF has been studied in many systems, primarily yeast, cultured cell lines, and the oocytes of both amphibians and marine invertebrates (for examples see Beach *et al.*, 1982; Gould & Nurse, 1989; Gautier and Maller, 1991; Meijer and Mordret, 1994; Abraham *et al.*, 1995; Eblen *et al.*, 1995; Meijer *et al.*, 1997). The aims of the studies here are to investigate the regulation and inhibition of MPF, particularly of the cdk1 subunit, during CMF-induced oocyte maturation in *Arenicola marina*.

7.2 MATERIALS AND METHODS

7.2.1 Solutions

Details of the antibodies used are provided in section 6.2.1. The buffers used are identical to those given in 6.2.1 in addition to:

Gel Filtration Buffer: 200mM NaCl, 0.1% Bij 35, 12.5mM β-glycero-phosphate, 12.5mM MOPS, pH 7.2, 0.5mM EGTA, 7.5mM $MgCl_2$, 1mM DTT, 0.1mM NaF, 1mM NaN_3

Lysis buffer: 1% Nonidet P-40, 1mM EDTA, 1mM DTT, 10 $\mu g.ml^{-1}$ leupeptin, 10 $\mu g.ml^{-1}$ soybean trypsin inhibitor and 100 mM benzamidine in phosphate-buffered saline (pH 7.2-7.4 140mM NaCl, 2.7mM KCl, 1.5mM KH_2PO_4 and 8.1mM Na_2HPO_4).

Tris-Buffered B (TBB): 50mM Tris, pH 8, 50mM NaCl, 1mM EDTA and 20mM DTT.

7.2.2 Methodology

The phosphorylation state of cdk1 was determined using a combination of 3 methods: observation of changes in electrophoretic mobility (for details see section 6.2.2); cross-reactivity with different antibodies (for details see section 6.2.2); *in vitro* incubation with different phosphatases.

7.2.3 Polychaetes and Oocytes

Arenicola marina

Gravid females of *Arenicola marina* were collected from beaches around Fife during the breeding season (see section 3.2.1). Prophase I and metaphase I oocytes were collected as described in section 3.2.2. Oocyte samples were placed into Eppendorf tubules (50 µl of packed oocytes) or into 15 ml centrifuge tubes (1 ml of packed oocytes) and frozen in liquid nitrogen (N_2). In addition, the oocytes were matured *in vitro* by incubation with CMF (see section 3.2.2) and then frozen in liquid N_2 every 20 minutes until maturation was complete (i.e. the oocytes reached metaphase I). The oocytes

were stored in a liquid N_2 biostore or a -70 °C or -80°C freezer, until ready for use.

Starfish

For details on obtaining prophase and metaphase I oocytes from starfish see section 6.2.3.

7.2.3 Purification, Gel Electrophoresis, Western Blotting and Histone H1 Kinase Assay.

cdk1/cyclin B was purified from the following samples of *Arenicola marina* oocytes: coelomic prophase I oocytes; spawned metaphase I oocytes and time-series of oocytes during CMF-induced maturation. In addition, cdk1/cyclin B was extracted from starfish oocytes for a positive control. Purification was achieved by affinity chromatography, as described previously in section 6.2.5. In addition, the methodology used for PAGE (polyacrylamide gel electrophoresis), Western blotting and kinase activity assay are all provided in section 6.2.6 and 6.2.7. The only difference is that after heating, only 30 µl of Laemmli sample buffer containing the recovered proteins, was loaded onto the gels.

7.2.4 Preparation and Purification of cdc25 and pyp3

Escherichia coli strains (donated by Drs K. Galaktionov and D. Beach) were transformed by plasmids encoding the gene fusion constructs of GST and yeast pyp3 or human cdc25A. A preculture was made by inoculating the bacteria in 100 ml of LB broth, containing 100 $\mu g.ml^{-1}$ of ampicillin, and placed at 37°C for 6-7 hours under constant rotation. Aliquots of the preculture were inoculated into larger volumes of LB broth (1 ml per 250 ml)

containing 100 $\mu g.ml^{-1}$ ampicillin and placed under constant rotation at 30°C until the appropriate culture density was reached (absorbence of 0.8 -1 at 600 nm). Once the correct density was obtained, 0.4mM isopropyl-1-thio-β-D-galactopyranoside was added and incubation of the culture continued for a further 8-10 hours. Following this, the bacterial culture was centrifuged (3000 x g for 15 min at 4°C) to collect the cells. The supernatant was removed and discarded, and the bacterial pellet was frozen at -80 °C until ready for use.

When ready for extraction, the bacterial pellets were placed in lysis buffer and fragmented in a Potter Homogeniser for 10 minutes. The bacterial suspension was then sonicated using a titanium probe (approximately 30 - 60 seconds per 40 ml of suspension). The cell lysis process (homogenisation followed by sonication) was repeated a further 2 to 3 times. The lysed-bacteria were then ultracentrifuged (100,000 x g) for 30 minutes at 4°C. The supernatant was kept and frozen in 10 ml aliquots, until ready for use.

Purification of the pyp3 and cdc25 phosphatases was achieved by affinity chromatography. One millilitre of glutathione-coated sepharose beads was added per 10 ml of extract and placed on a rotator for 30 minutes at 4°C. The beads were then washed in lysis buffer (x 4) followed by washing in Tris-buffer B, TBB (x 4). The proteins were eluted by incubation of the beads with 20 mM glutathione in TBB (500 µl of 20 mM glutathione per 1 ml of beads) and placed onto a rotator at 4°C for 15 minutes. Following this stage and after a brief centrifugation, the supernatant (now containing the phosphatases) was removed and frozen rapidly at -30°C. To assay the activity of the purified phosphatases, 20 µl of the phosphatase was added to 20 µl of 100mM DTT (made up in TBA) and 140 µl of TBA. The samples were incubated at 37°C for 15 minutes. Following this period, 20 µl of 500mM *p*-nitrophenylphosphate in TBA was added and incubated for

another 30 minutes. Phosphatase activity was signified by the sample turning a dark yellow colour.

7.2.5 *In Vitro* Incubation with Phosphatases

cdk1/cyclin B was purified from prophase I and metaphase I oocytes onto $p9^{CKhs1}$ sepharose beads as described before (see section 6.2.4). The final bead buffer wash was removed and replaced with 100 µl of TBA (control) or a sample of one of the following phosphatases; 100 µl of pyp3; 100 µl of cdc25; 20 µl of acid phosphatase; 20 µl of alkaline phosphatase (all made up in TBA plus protease inhibitors to limit degradation). The samples were incubated in a waterbath at 30°C for 30 minutes, during which time each sample was mixed briefly every 2 minutes. Following incubation the samples were placed on ice and the phosphatatases and control solutions removed and replaced with 1 ml of TBA. The beads were washed 3 more times in TBA, before washing the beads in bead buffer (x 4). cdk1/cyclin B from each sample was assayed for histone H1 kinase activity as described before (see section 6.2.6). Following kinase activity assessment, the proteins were then recovered by the addition of 50 µl of Laemmli sample buffer and heated for 3 minutes. The samples were run on SDS-PAGE, blotted and analysed, as described previously (see section 6.2.5).

7.2.6 Gel Filtration

Five millilitres of metaphase oocytes in homogenisation buffer were sonicated using a titanium probe. The oocyte extract was loaded on a 100 x 2.6 cm column packed with Sepharose S-200HR and equilibrated with degassed gel filtration buffer. It was calibrated with Bio-Rad gel filtration molecular weight markers before and after gel filtration of the oocyte extract. 6 ml/10 minute fractions were collected and following gel filtration, 1 ml fractions were loaded onto 10 µl of p9 beads as described above (see section

6.2.4). The bound proteins were assayed for kinase activity and then recovered and run on SDS-PAGE, Western blotted and analysed as described previously.

7.2.7 *In Vitro* Incubation with cdk1/Cyclin B Inhibitors

Three microlitres of prophase I oocyte samples were incubated *in vitro* in 50 µl of CMF for 30 minutes and then washed in TFSW before placing in various concentrations of olomoucine (stock made up in DMSO), roscovitine (stock made up in TFSW) or TFSW (control). The oocytes were incubated for at least 4 hours and scored for germinal vesicle breakdown (GVBD).

cdk1/cyclin B was purified by affinity chromatography from metaphase oocytes, as described before (see section 6.2.4), and incubated with various concentrations of roscovitine or olomoucine. Following this, each sample was assayed for kinase activity using histone H1 and radioactive ATP, as described previously (section 6.2.6).

7.3 Results

7.3.1 Controls

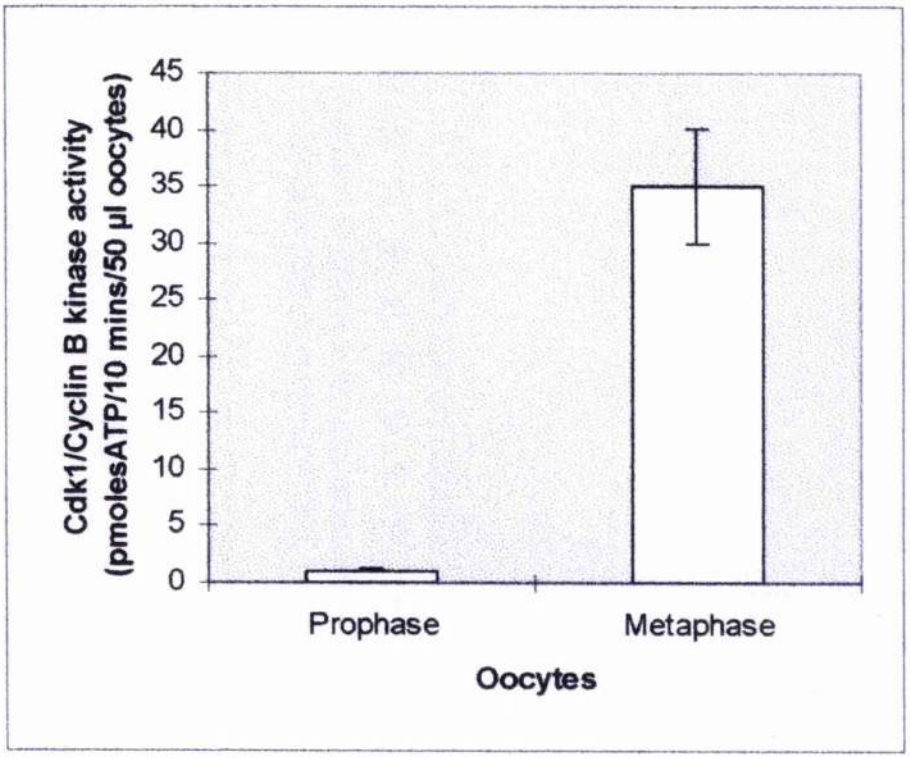
Starfish cdk1/cyclin B was used as a control throughout experimentation (for details see section 6.3.1). In addition, the anti-PSTAIRE amd anti-GEGTYG antibodies were tested cross-reacted with the peptides they were raised against (see section 6.3.1).

7.3.2 Activation of cdk1/Cyclin B

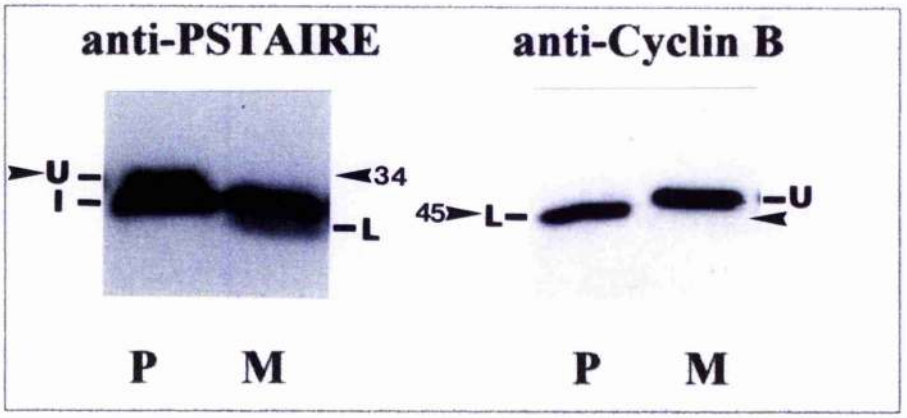
There were two forms of cdk1 present in the prophase I oocytes of *Arenicola marina* a large pool of cdk1 in the intermediate position and a smaller pool of cdk1 observed as an upper form (fig. 7.1B). During the

Figure 7.1: A) Mean kinase activity of cdk1/cyclin B, purified from *Arenicola marina* oocytes, by measuring the amount of radioactive phosphate incorporated into histone H1 (vertical bars show standard error of the mean). B) Western blots of MPF subunits from prophase I (P) and metaphase I (M) oocytes of *Arenicola marina.* cdk1, cross-reacted with anti-PSTAIRE (U = 35 kDa); cyclin B, cross-reacted with anti-cyclin B (L = 45 kDa). **U**, upper form; **I**, intermediate form; **L**, lower form.

A


Cdk1/Cyclin B kinase activity
(pmolesATP/10 mins/50 µl oocytes)
45
40
35
30
25
20
15
10
5
0
Prophase
Metaphase
Oocytes

B


anti-PSTAIRE
anti-Cyclin B
U
I
34
L
45
L
U
P
M
P
M

prophase to metaphase transition, the upper band is converted to a lower form, indicating that dephosphorylation had occurred (Fig. 7.1B and 7.2A). In contrast, cyclin B is converted to a more electrophoretically retarded form and observed as an upper form once maturation is complete (Fig. 7.1B). The conversion from a lower to an upper form indicates that phosphorylation has occurred. These changes in the electrophoretic mobility of the MPF protein subunits was accompanied by activation of cdk1/cyclin B as demonstrated using the histone H1 kinase assay (Fig. 7.1A).

The dephosphorylation of cdk1 during meiotic maturation was confirmed in Figs 7.2B and 7.2C which show changes in cdk1 during oocyte maturation, cross-reacted with anti-phosphotyrosine and anti-GEGTYG respectively. The upper band was tyrosine-phosphorylated and became dephosphorylated (Fig 7.2B), increased in electrophoretic mobility to form the lower band that then cross-reacted with anti-GEGTYG (Fig. 7.2C). In addition, incubation with acid and alkaline phosphatase (data not shown) and pyp3 (Fig. 7.3B) results in the disappearance of the upper band demonstrating that this band was indeed phosphorylated.

The larger pool of cdk1, positioned at the intermediate level on Western blots (Figs 7.1B and 7.2A) undergoes no change in electrophoretic mobility during the prophase to metaphase transition and is recognised by the same antibody-type (anti-PSTAIRE and anti-GEGTYG) in prophase I oocytes, throughout maturation and in the metaphase I oocytes (Fig. 7.1B; 7.2A and C). Hence there was no change in phosphorylation state of this intermediate band during meiotic reinitiation. Furthermore this band did not cross-react with the anti-phosphotyrosine (Fig. 7.2B), showed excellent binding with anti-GEGTYG (Fig 7.2C) and showed no changes occurred following incubation with any of the phosphatases tested (data not shown). This indicates strongly that this protein is unphosphorylated.

Figure 7.2: Western blots of the cdk1 subunit from *Arenicola marina* oocytes during the prophase to metaphase transition, cross-reacted with A) anti-PSTAIRE, B) anti-phosphotyrosine and C) anti-GEGTYG antibodies. **U**, upper form (35 kDa); **I**, intermediate form; **L**, lower form.

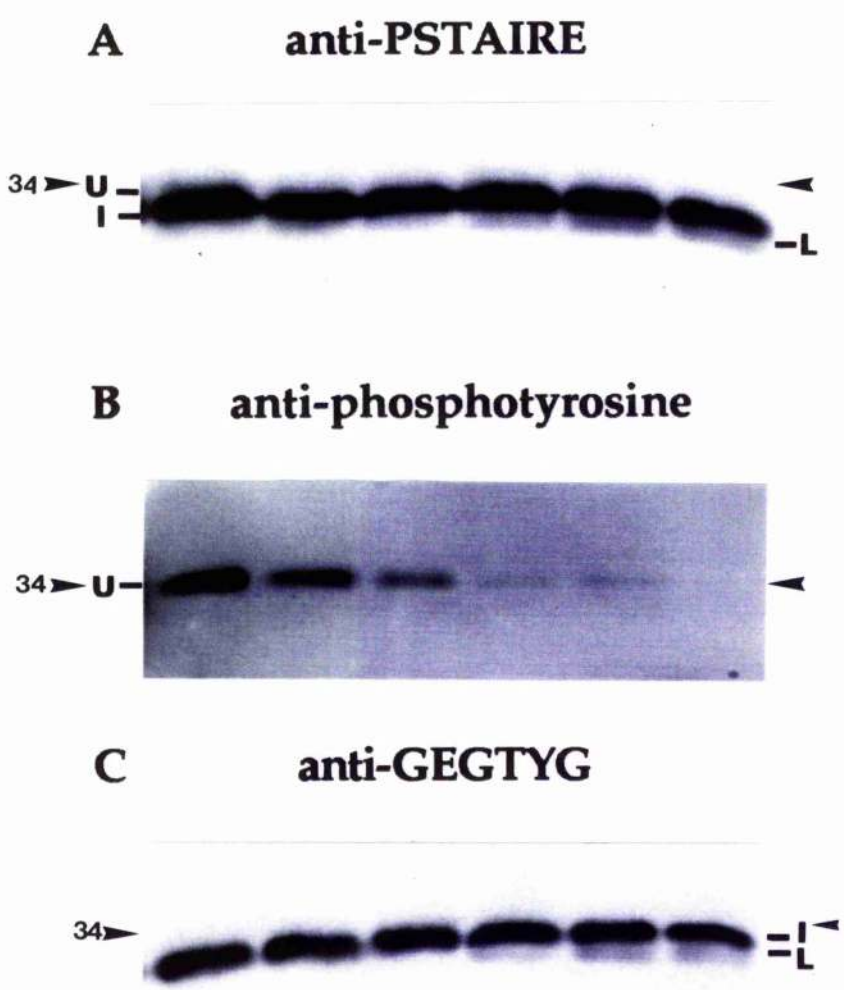
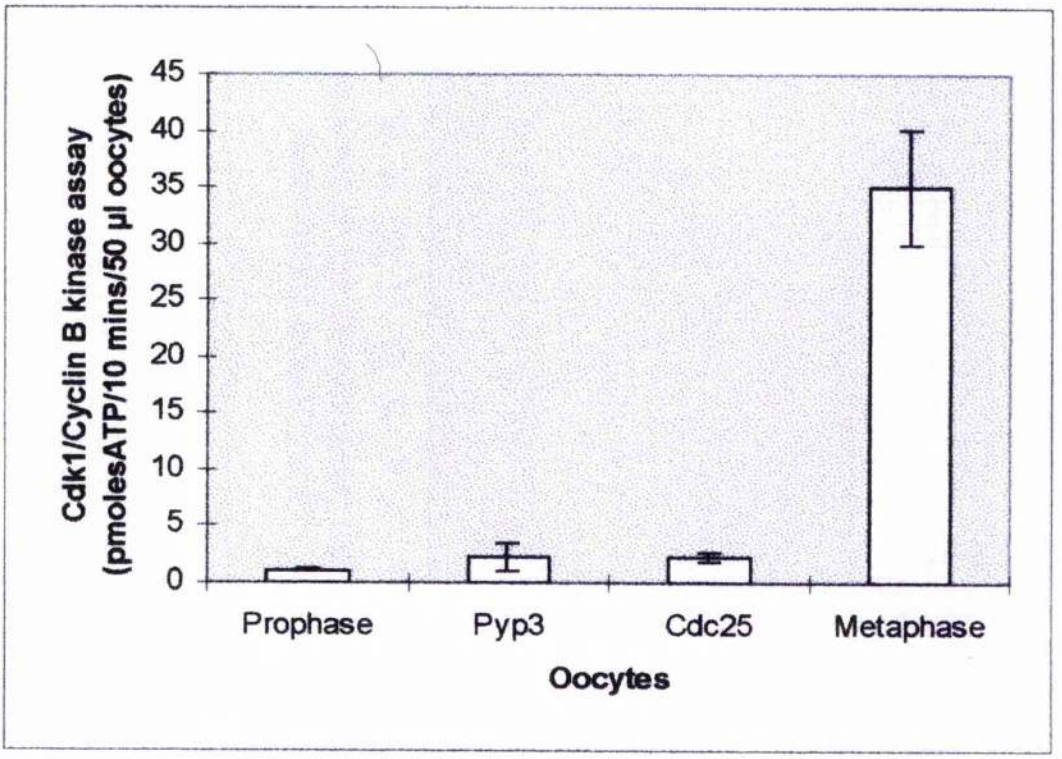

A
anti-PSTAIRE
34
U
I
L
B
anti-phosphotyrosine
34
U
C
anti-GEGTYG
34
I
L
0
20
40
60
80
100
oocyte maturation (mins)

Figure 7.3: A) Mean kinase activity of cdk1/cyclin B purified from *Arenicola marina* prophase I and metaphase I oocytes, and of cdk1/cyclin B (from prophase I oocytes) following incubation with pyp3 and cdc25 phosphatases. B) Western blots of cdk1 subunits cross-reacted with anti-phosphotyrosine from *Arenicola marina* prophase I (P) and metaphase I (M) oocytes, and cdk1 (from prophase I oocytes) following incubation in pyp3 and cdc25 phosphatases. **U** = upper form (35 kDa).

A


Cdk1/Cyclin B kinase assay
(pmolesATP/10 mins/50 µl oocytes)
45
40
35
30
25
20
15
10
5
0
Prophase
Pyp3
Cdc25
Metaphase
Oocytes

B

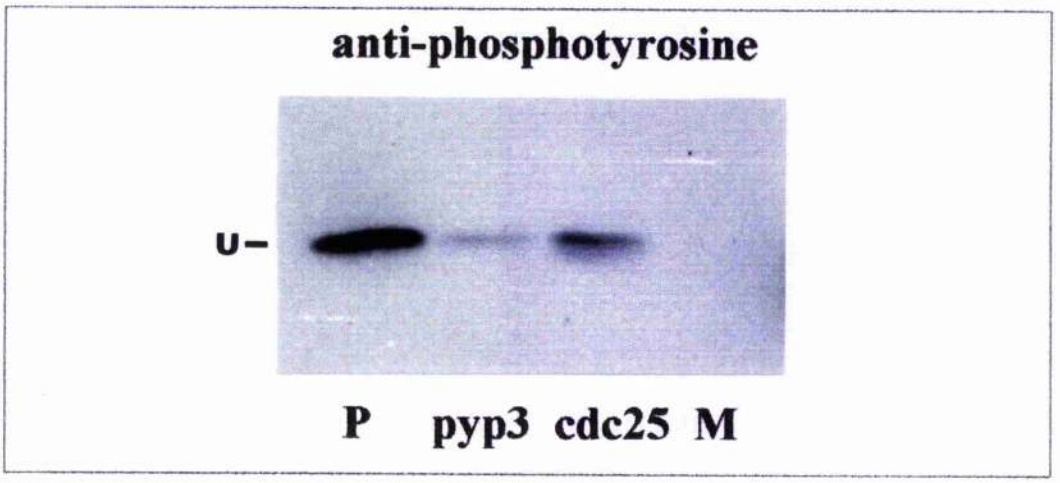

anti-phosphotyrosine
U–
P
pyp3
cdc25
M

Figure 7.4: Gel filtration of cdk1 and cyclin B purified from *Arenicola marina* metaphase I oocytes. The molecular weight standards are provided in kDa. The cdk1/cyclin B kinase activity of each of the fractions is given. The Western blots show cdk1 (cross-reacted with anti-PSTAIRE) and cyclin B (cross-reacted with anti-cyclin B) from each of the fractions.

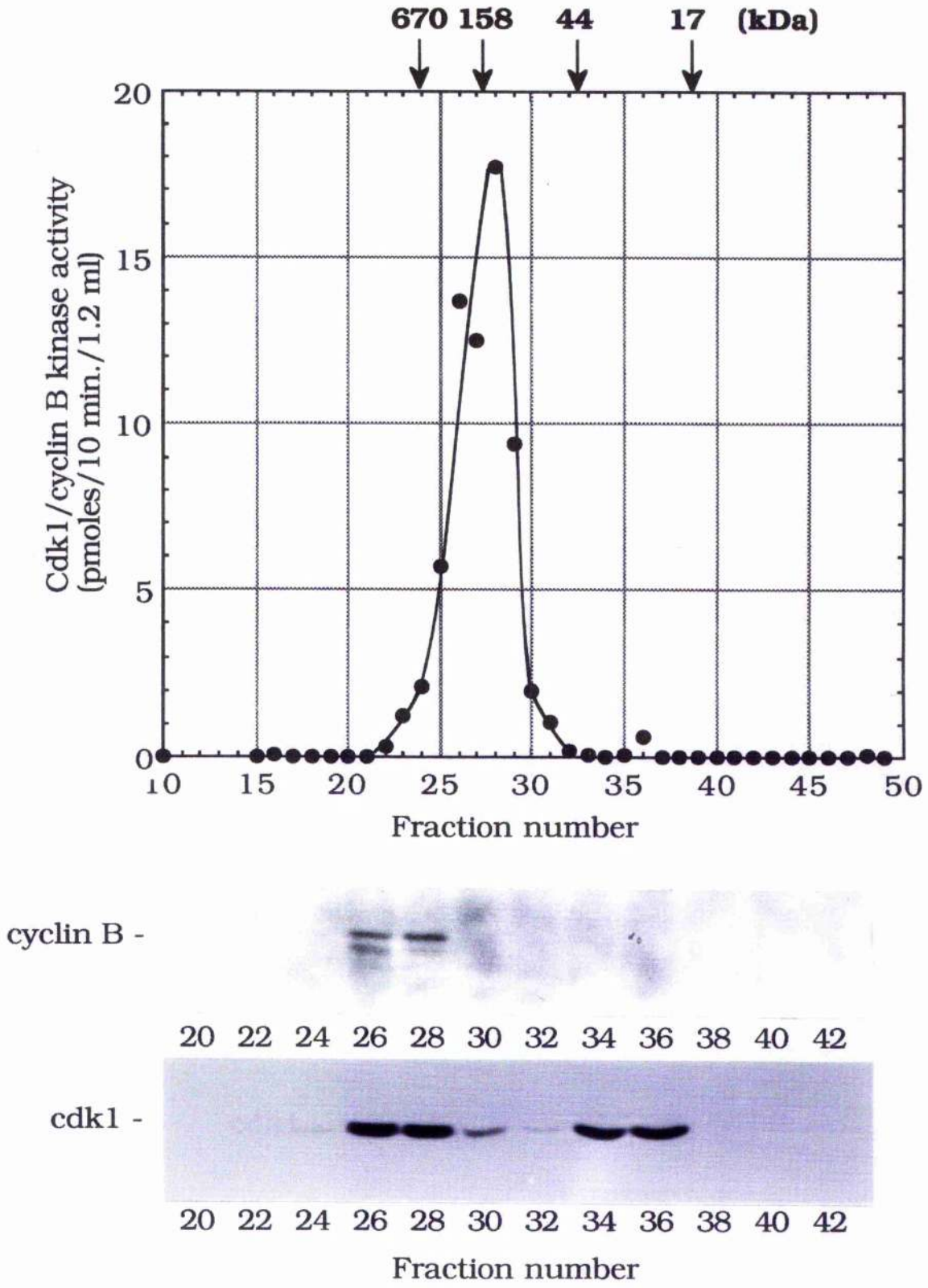
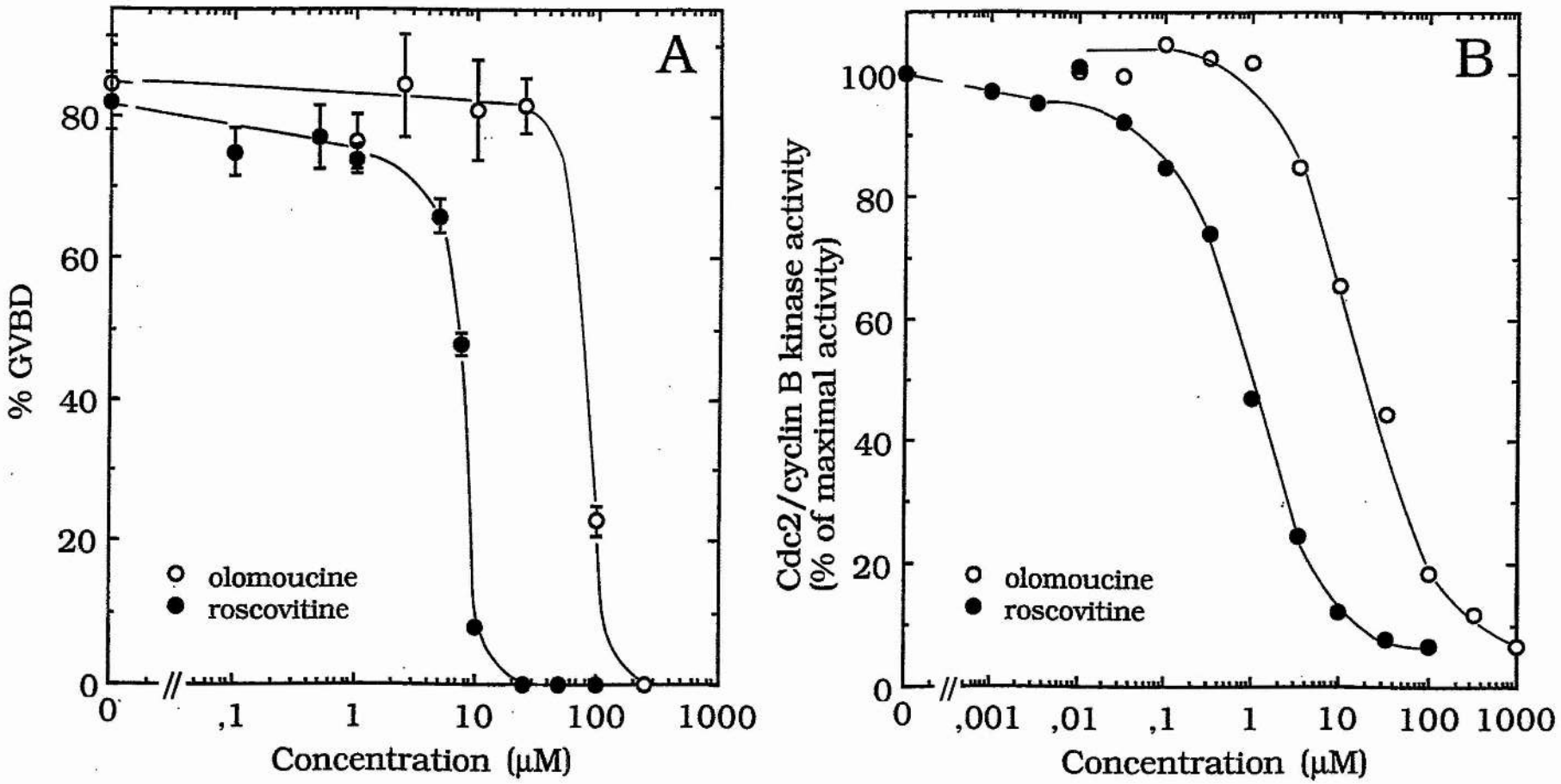

670 158 44 17 (kDa)
Cdk1/cyclin B kinase activity
(pmoles/10 min./1.2 ml)
0
5
10
15
20
10 15 20 25 30 35 40 45 50
Fraction number
cyclin B -
20 22 24 26 28 30 32 34 36 38 40 42
cdk1 -
20 22 24 26 28 30 32 34 36 38 40 42
Fraction number

Figure 7.5: Roscovitine and olomoucine dose-dependent curves for inhibition of A) GVBD in maturing *Arenicola marina* oocytes and B) active cdk1/cyclin B purified from metaphase I oocytes.


A
% GVBD
80
60
40
20
0
olomoucine
roscovitine
0
,1
1
10
100
1000
Concentration (µM)
B
Cdc2/cyclin B kinase activity
(% of maximal activity)
100
80
60
40
20
0
olomoucine
roscovitine
0
,001
,01
,1
1
10
100
1000
Concentration (µM)

potent inhibitor of the two causing 50% inhibition of GVBD at a concentration of 8μM (IC_{50}) and olomoucine induced an IC_{50} of GVBD at 50 μM (Fig. 7.5A). In addition, active cdk1/cyclin B was purified from metaphase oocytes and incubated with various concentrations of olomoucine and roscovitine. Results demonstrated that these chemicals directly inhibited the kinase activities of the cdk1/cyclin B complex (Fig. 7.5B).

7.4 DISCUSSION

Cyclin B is already present in prophase I oocytes of *Arenicola marina* and hence its synthesis is not required for MPF activation. As with the oocytes of starfish, *Xenopus* and mammals (see section 1.5.1), activation of MPF in the oocytes of *A. marina*, involves dephosphorylation of the cdk1 subunit. In contrast, the results demonstrate that only a small pool of the total cdk1 present in *A. marina* oocytes is used during meiotic maturation of the oocytes. This small pool cdk1 was tyrosine-phosphorylated at prophase I and becomes dephosphorylated during meiotic maturation. The second larger pool of cdk1 is unphosphorylated and undergoes no change in electrophoretic mobility or cross-reactivity with antibodies during oocyte maturation. Hence it appears that this pool of cdk1 is not used, altered or activated during meiotic maturation.

Results from the gel filtration experiment reveal that cdk1 is present in two forms; complexed with cyclin B and as a monomer. Evidence derived from other studies demonstrate that phosphorylation of cdk1 does not occur until association with cyclin B takes place (Solomon *et al.*, 1990; Meijer, et al., 1991; Parker *et al.*, 1991). It is likely, therefore, that the small pool of tyrosine-phosphorylated cdk1 is associated with cyclin B but the intermediate unphosphorylated form is monomeric. The purpose of the excess

monomeric cdk1 remains unclear but it could function as a store, to be utilised later in development, i.e. for mitotic divisions during embryogenesis. A similar scenario has been described in sea urchin oocytes (Meijer *et al.*, 1989a; Meijer *et al.*, 1991). In sea urchin oocytes only a fraction of the cdk1 is tyrosine phosphorylated, bound to cyclin B and utilised at M-phase and surplus cdk1 is monomeric and remains inactive on the transition to M-phase (Meijer *et al.*, 1989a; Meijer *et al.*, 1991). It should be noted, however, that sea urchin oocytes arrest at post-meiotic prophase, i.e. once the female pronucleus has formed. Hence, dephosphorylation of tyrosine on the complexed cdk1 subunit results in MPF activation which leads to direct entry into mitosis during embryo formation and not meiotic maturation as is the case with *A. marina*. Hence, although *A. marina* oocyte maturation and fertilization shows few similarities with sea urchins, MPF regulation is comparable.

In porcine prophase I oocytes, there is a small amount of cyclin B present that is complexed to cdk1 and during maturation *de novo* cyclin B synthesis occurs and recruits the excess monomeric cdk1 (Wu *et al.*, 1997). It is plausible that cyclin B synthesis also occurs during meiotic maturation in *Arenicola marina* oocytes, and recruits the monomeric cdk1 to form active MPF. However, observation of Western blots of cyclin B from *A. marina* prophase I and metaphase I oocytes revealed no changes in the amount of this protein.

Arenicola marina and many mollusc species (for example, *Mytilus, Patella, Ruditapes*) share the same pattern of oocyte maturation (Abdelmajid *et al.*, 1993b; Néant *et al.*, 1994; Watson and Bentley, 1997). In both cases, following release of the prophase block by an extracellular trigger, oocyte maturation is once more interrupted at metaphase I until fertilization occurs. Data using the molluscan oocyte model shows that release from metaphase I block and

subsequent entry into anaphase is induced by the destruction of cyclin B, which inactivates MPF, and allows the cell to exit from M-phase (Abdelmajid *et al.*, 1993b; Néant *et al.*, 1994). In addition, emetine treatment (which inhibits protein synthesis) induces molluscan metaphase I arrested oocytes to complete meiosis (Abdelmajid *et al.*, 1993b; Néant *et al.*, 1994). It is thought that the emetine treatment prevents the synthesis of proteins which normally prevent cyclin proteolysis (Colas *et al.*, 1993). Hence it is also possible that the metaphase block maintained in *Arenicola* oocytes is due to the prevention of cyclin B destruction. The results from this study support this hypothesis, as cyclin B is not destroyed at metaphase and MPF is still active.

Pre-MPF, from *Arenicola marina* prophase oocytes, was incubated with pyp3 and resulted in tyrosine-dephosphorylation of cdk1 yet caused little effect in kinase activity. This was most likely to have occurred due to double inhibitory phosphorylations upon cdk1. Hence following pyp3 phosphatase activity upon tyrosine 15 (or equivalent), full activation is blocked due to threonine 14 (or equivalent) remaining phosphorylated. Further evidence is revealed by observation of the blots as pyp3 did not produce the fully dephosphorylated lower form of cdk1. This scenario has also been described using starfish oocytes, whereby pyp3 acts on pre-MPF to form the low kinase activity intermediate form of threonine (T) 14 dephosphorylated, tyrosine (Y) 15 phosphorylated cdk1, "T-Yp". In addition, anti-GEGTYG antibodies (which will also bind to T-Yp, Borgne and Meijer, 1996) did not cross-react with the upper form of cdk1 indicating that both threonine and tyrosine are phosphorylated at prophase in the oocytes of the lugworm *Arenicola marina.* It appears, therefore, that as with starfish and other models, the inactive form of cdk1 (upper migrating form) in *A. marina* oocytes has the dual threonine 14 and tyrosine 15 (or the equivalent) inhibitory residues.

In contrast to expectations, cdc25 causes only a partial

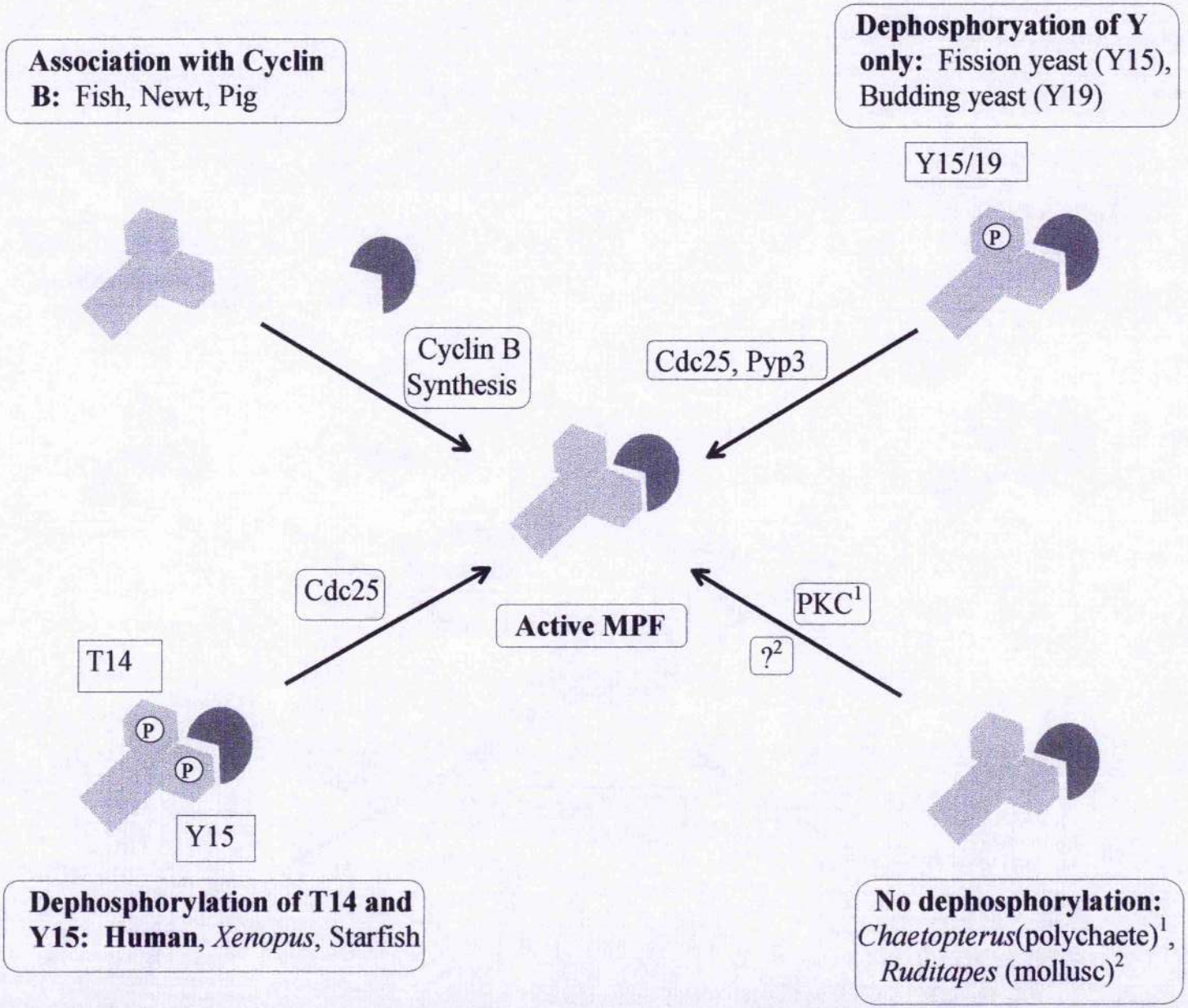
dephosphorylation of the upper form, and did not cause any increase in kinase activity. These results conflict those found with respect to the starfish oocytes model whereby *in vitro* incubation of pre-MPF with cdc25 causes complete dephosphorylation and activation of cdk1/cyclin B (Borgne and Meijer, 1996). The reason for its ineffectiveness in *Arenicola* oocytes is uncertain and further experiments should be carried out in future work. One possible explanation includes an inhibitor of cdc25 that is physically associated with the cdk1/cyclin B complex, prevents cdc25 reaching the tyrosine and threonine residues until ready for activation.

Previous reports demonstrate that olomoucine and roscovitine are able to prevent meiotic reinitiation in the oocytes of starfish, mollusc, amphibian and mouse (Abraham *et al.*, 1995; Meijer *et al.*, 1997). The results presented here show that this is also true in the oocytes of the polychaete *Arenicola marina*. Olomoucine and roscovitine inhibit GVBD in *Arenicola marina* oocytes, which were previously treated with CMF, at an IC_{50} of 50μM and 8μM, respectively. Both these chemicals are selective with respect to inhibition of cdk's and previous results have shown that these chemicals will successfully inhibit cdk1/cyclin B (Vesely *et al.*, 1994; Meijer *et al.*, 1997). It is conceivable, therefore, that inhibition of GVBD in *Arenicola* by these two chemicals is due to the inhibition of cdk1/cyclin B activity. Evidence supporting this hypothesis is provided by *in vitro* incubation of purified active cdk1/cyclin B (from *Arenicola* metaphase I extracts) which demonstrated that both olomoucine and roscovitine directly inhibit the kinase activity in a dose-dependent manner. Roscovitine is also shown to be the most potent inhibitor of the two chemicals, as is the case in other studies (De Azevedo *et al.*, 1996). Further investigations should be carried out to determine precisely why these chemicals are able to inhibit kinase activity and why one is more potent than the other. Studies such as these could lead, in time, to the manufacture of novel cdk inhibitors with increased

selectivity and potency and could provide a base on which to model tumour-suppressing pharmaceuticals.

In summary, the form that cdk1 adopts to remain inactive prior to M-phase can be divided into four main categories (see Fig. 7.6). The mechanism by which MPF activation is achieved is irrespective of phylum or the stage at which the oocytes arrest. The results produced from the studies described here indicate that only a small portion of cdk1 is utilised during meiotic re-initiation in *Arenicola marina* oocytes. This form is phosphorylated at threonine 14 and tyrosine 15 (or equivalent residues) and bound to cyclin B. Dephosphorylation of the two residues induces activation and M-phase entry. A larger pool of cdk1 is maintained as a monomer to prevent activation as it is not required for meiotic maturation but could be utilised during later divisions during embryogenesis (see Fig. 7.7).

Figure 7.6: Diagrammatic representation of MPF regulation and activation in different organisms.


Association with Cyclin B: Fish, Newt, Pig
Dephosphoryation of Y only: Fission yeast (Y15), Budding yeast (Y19)
Y15/19
P
Cyclin B Synthesis
Cdc25, Pyp3
Cdc25
Active MPF
PKC[1]
?[2]
T14
P
P
Y15
Dephosphorylation of T14 and Y15: Human, Xenopus, Starfish
No dephosphorylation: Chaetopterus(polychaete)[1], Ruditapes (mollusc)[2]

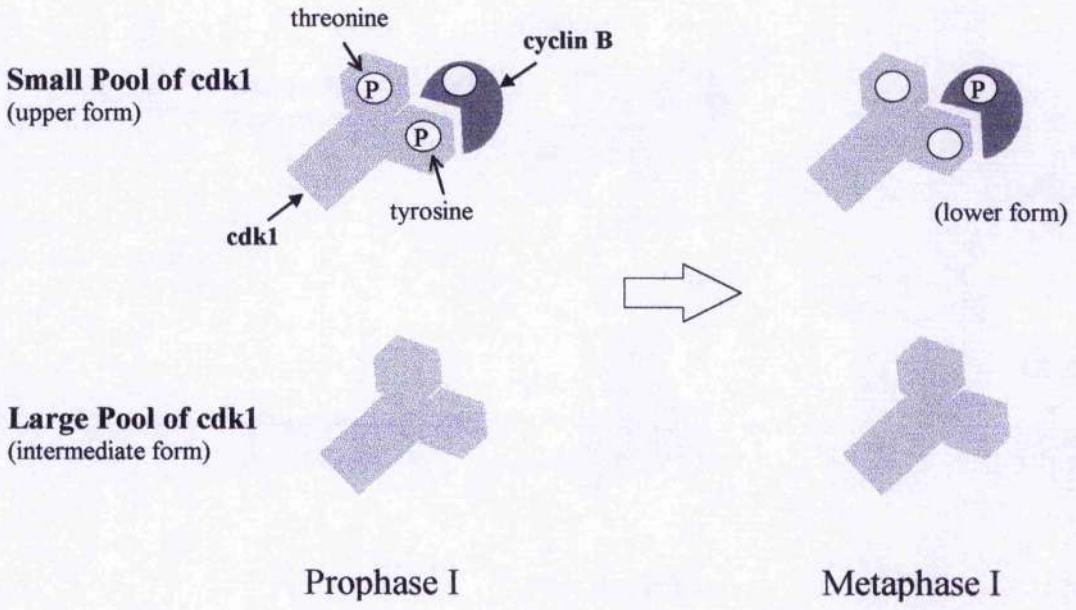


Figure 7.7. Diagrammatic summary of MPF regulation and activation in *Arenicola marina* oocytes during meiotic maturation. The prophase oocytes contain a small pool of phosphorylated cdk1 which is bound to cyclin B and becomes dephosphorylated during the prophase I to metaphase I transition (Fig. 7.2). In addition, a larger pool of cdk1 which is unphosphorylated and monomeric is present but is not utilised during meiotic maturation. Results indicate that cyclin B becomes phosphorylated (Fig. 7.1).

CHAPTER 8

GENERAL DISCUSSION

8.1 INTRODUCTION

The majority of the research presented in this thesis was directed towards the lugworm *Arenicola marina*, from the hormonal cascade that triggers oocyte maturation to the activation of the M-phase promoting factor. Each chapter on *A. marina* deals with a specific aspect of this cascade, but together they provide a sequential account of oocyte maturation in this species. To compare and contrast, work was also carried out on oocyte maturation in the closely related species, *A. defodiens* and the king ragworm *Nereis virens* .

8.2 ULTRASTRUCTURE OF MEIOTIC MATURATION IN THE OOCYTES OF *ARENICOLA MARINA* AND *NEREIS VIRENS*

Meiotic maturation in *Arenicola marina* and *Nereis virens* was characterised using transmission electron microscopy. Marine invertebrate oocytes and eggs are difficult to fix, as reported by previous authors (for example see Eisenman and Alfert, 1982). This is especially true in fertilized nereid oocytes due to the post-insemination extracellular jelly layer that forms and restricts fixative penetration leading to poor morphological preservation (Bass and Brafield, 1972). An alternative method to conventional fixation was described "microwave assisted fixation" (MAF). MAF produced superior morphological fixation of fertilized *N. virens* oocytes, including delicate organelles, and primary fixation was completed in 10 - 12 seconds, as opposed to an hour or more. MAF has been used on many tissues (Login and Dvorak, 1993) nevertheless, this is the first time that this technique has been used for the fine morphological preservation of marine invertebrate eggs. Unfertilized and fertilized gametes from *A. marina*

were fixed using conventional chemical fixation.

There were both similarities and differences in the ultrastructure of meiotic maturation of the two polychaete species. Release from the prophase I block, by fertilization in *Nereis virens* oocytes and CMF in *Arenicola marina* oocytes, resulted in cortical granule exocytosis and germinal vesicle breakdown (GVBD). Cortical granule exocytosis is more commonly associated with fertilization, hence the occurrence of this event during hormone induced maturation in *A. marina* oocytes was unusual. Furthermore, cortical granule exocytosis does not occur during the prophase I to metaphase I transition in the oocytes of the closely related species *A. defodiens* (Meijer, 1979b). This reveals yet another difference between the two *Arenicola* species (see also Watson *et al.*, 1998).

The most obvious ultrastructural difference between the two species was that fertilized *N. virens* oocytes produced a thick extracellular jelly coating, which is common throughout the Nereidae. In the absence of this jelly coat, development proceeds normally in *N. virens* embryos (Bass and Brafield, 1972) indicating that polyspermy was avoided (which would have lead to abnormal development). This provides circumstantial evidence that the function of the jelly layer is not to prevent multiple inseminations. Furthermore, one would reason that the extremely thick jelly layer that forms is rather excessive if its role is to act as a polyspermic block. Section 2.4 discusses the hypothesis that this extracellular coating is formed to cushion and protect the developing embryo from the rigours of the external environment. It is conceivable that because the fertilized oocytes and developing embryos of *Arenicola marina* are maintained in the maternal burrow for some time, a protective coating is not required. In contrast, those of *Nereis virens* need an extra covering as they are found on the sediment surface.

8.3 Regulation of Oocyte Maturation in *Arenicola marina*.

Howie (1963, 1966) determined that the release of the prostomial maturation hormone (PMH) leads to oocyte maturation in *Arenicola marina*. Watson (1997) demonstrated that release of PMH involves a hormonal cascade that leads to the production of the active coelomic maturation factor (CMF) which acts upon the oocyte. The research from this thesis shows that CMF then triggers the activation of MPF within the oocyte cytoplasm to induce maturation (Fig. 8.1).

The studies in this thesis revealed that PMH was greater than 10 kDa and the signal transduction pathway from PMH to CMF activation was investigated but results were inconclusive. Due to the size of CMF (> 30 kDa, Watson and Bentley, 1998a) it is highly probable that it acts extracellulary upon the oocyte surface, most likely via G-proteins as demonstrated in the oocytes of other organisms (see section 1.3.1). The second messenger that relays the CMF signal to the activation of MPF was also investigated. The combined results of this thesis and previous studies by Watson (Watson *et al.*, 1998) demonstrate that neither a change in calcium levels or pH are the second messengers involved during the release of the prophase I block.

8.4 M-phase Promoting Factor, MPF in Polychaete Oocytes

In prophase I oocytes, progression to M-phase is prevented through the inhibition of MPF activity by one of two principal mechanisms. In the prophase I oocytes of starfish, *Xenopus* and most mammals, cdk1 and cyclin

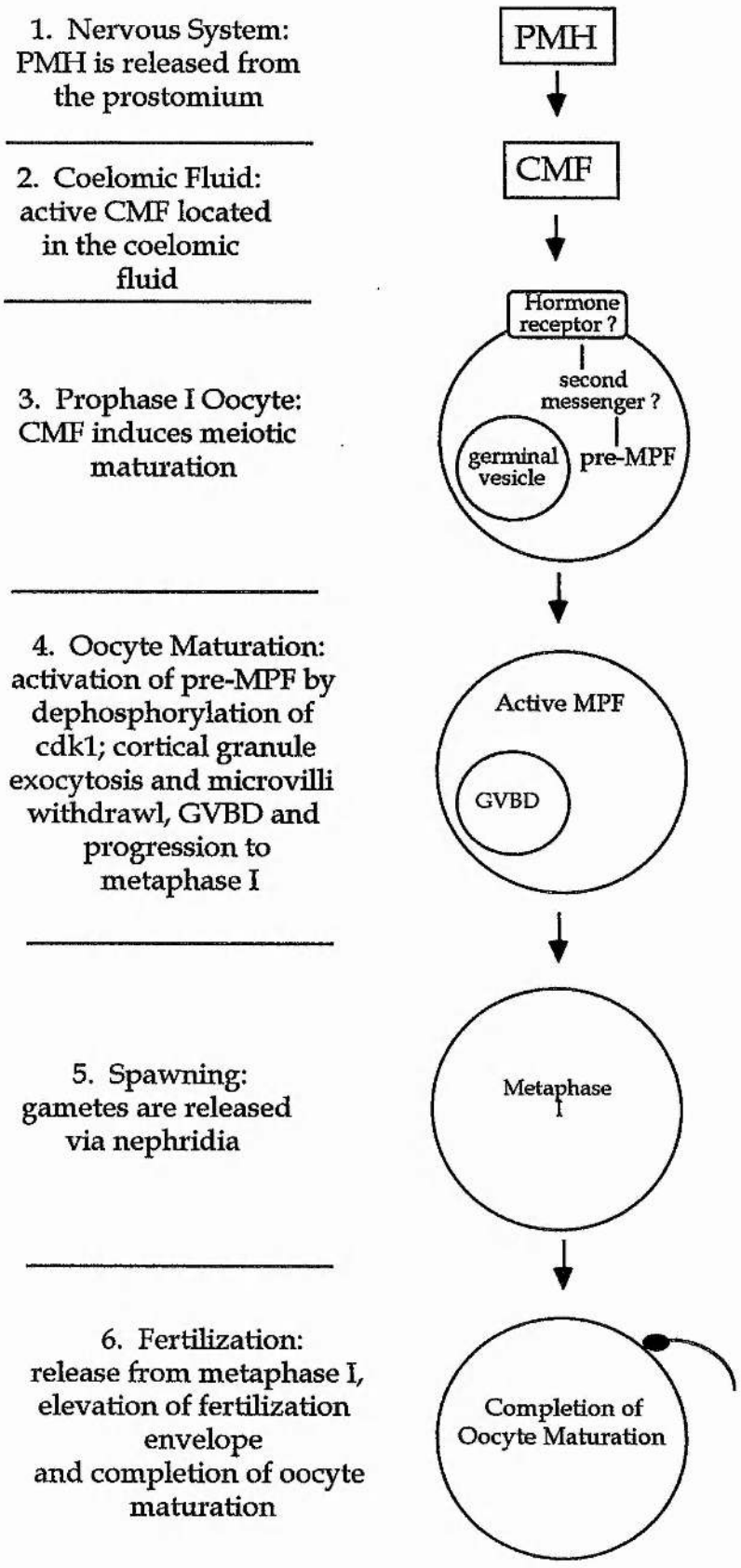


Figure 8.1: Oocyte maturation in *Arenicola marina*

B are maintained in a precursor complex ("pre-MPF") which is activated post-translationally by dephosphorylation of the cdk1 subunit (see section 1.5.1). By contrast, the prophase I oocytes of fish, newt and pig contain monomeric cdk1, and activation is achieved by the synthesis of cyclin B (see section 1.5.2). The results from this thesis demonstrated that the prophase I oocytes of *Arenicola marina* and *Nereis virens* contained pre-MPF, hence no cyclin B synthesis was required. Furthermore, MPF activation was achieved at meiotic reinitiation by post-translational dephosphorylation of cdk1 subunit. Differences, however, were noted with respect to MPF regulation in the oocytes of the two polychaete species, when compared to the "standard" post-transitional activation model (see section 1.5.1). In *A. marina* oocytes, only a fraction of cdk1 was phosphorylated and complexed with cyclin B and only this fraction was activated by dephosphorylation during meiotic maturation. Results indicated that the second larger pool of cdk1 was unphosphorylated, monomeric and not utilised during meiotic reinitiation but was possibly stockpiled for future mitotic divisions. In *N. virens* oocytes, only one pool of cdk1 was present, which was phosphorylated and complexed to cyclin B. In all higher eukaryotic organisms, however, the precursor of MPF is maintained inactive by the phosphorylation of both threonine 14 and tyrosine 15 on the cdk1 subunit (see section 1.5.1). Results, here, suggested that pre-MPF in *N. virens* oocytes contained tyrosine-only phosphorylated cdk1. The only other organism in which this has been recorded is yeast (see section 1.5.1).

Prior to the research presented in this thesis, cdk1/cyclin B activation during meiosis reinitiation had only been documented in one other polychaete, *Chaetopterus pergamentaceus* (Eckberg *et al.*, 1996; Eckberg, 1997). Pre-MPF complexes are present within the prophase I oocytes of this species, but cdk1 is not tyrosine phosphorylated and activation is not triggered by dephosphorylation (Eckberg *et al.*, 1996). As indicated by the results of

Eckberg (1996), cdk1/cyclin B in *C. pergamentaceus* is activated directly by protein kinase C (PKC), possibly through the phosphorylation of threonine 161. This residue, which must be phosphorylated in the active cdk1/cyclin B complex in all organisms (see section 1.5.3), was not investigated during the research presented in this thesis. Nevertheless, cdk1 in the pre-MPF of *Nereis virens* and *Arenicola marina* oocytes was tyrosine phosphorylated and activation occurred in conjunction with dephosphorylation, and therefore shows distinct differences from that of *Chaetopterus* sp.

In all the asteroids echinoderms studied to date, the mechanism of MPF regulation is the same, irrespective of the species (Meijer and Mordret, 1994). The regulation and activation of MPF is, however, clearly not ubiquitous throughout the class Polychaeta. Nevertheless, the 3 species (*Nereis virens, Arenicola marina* and *Chaetopterus pergamentaceus*) belong to different families within this class and more closely related species are more likely to have similar MPF mechanisms. Furthermore, inter-species variability is not solely confined to the polychaetes as MPF regulation in the two bivalves *Ruditapes philippinarum* and *Spisula solidissima* also exhibit differences. In both these mollusc species, cdk1 in the pre-MPF complexes is tyrosine phosphorylated, however, during activation dephosphoryation only occurs in *Spisula* oocytes (Abdelmajid *et al.*, 1994).

8.5 Regulation of oocyte maturation in the three polychaete species.

Differences in the oocyte maturation between *Nereis virens* and the two *Arenicola* species were to be expected, as they come from different families and are fertilized at different stages. However, differences between the two closlely related species *A. marina* and *A. defodiens* were surpising.

Figures 8.1, 8.2 and 8.3 provide a summary diagram of oocyte maturation in the three species.

8.6 FUTURE WORK

The next section describes future work on the regulation of the cell cycle which deserves investigation, and discusses why polychaete oocytes are such an apt model to investigate these processes.

Acquisition of Fertilizability

Oocyte maturation in many animals is associated with the acquisition of fertilizability. Prophase I oocytes of most species are "immature" (i.e. are unable to become fertilized) until maturation occurs. What changes occur during maturation that allows fertilization to occur? There are certain advantages to using *Arenicola marina* as opposed to starfish oocytes as a model to investigate the acquisition of fertilizability. Starfish oocytes do not undergo a second meiotic block after hormone induction and furthermore, the prophase I oocytes show a partial activation response to sperm (Miyake and Hirai, 1979) and hence do not provide a very good system for this study. On the other hand, *A. marina* prophase I oocytes undergo no activation response to sperm but show a complete fertilization response in metaphase I arrested oocytes. The oocytes of *A.marina* therefore provide an ideal system in which to investigate and answer such questions as:

- What changes occur within the oocyte during the prophase I to metaphase I transition that permits fertilization?
- Is the acquisition of fertilizability linked to the activation of protein kinases such as MPF, or possibly another kinase (e.g., MAPK)?

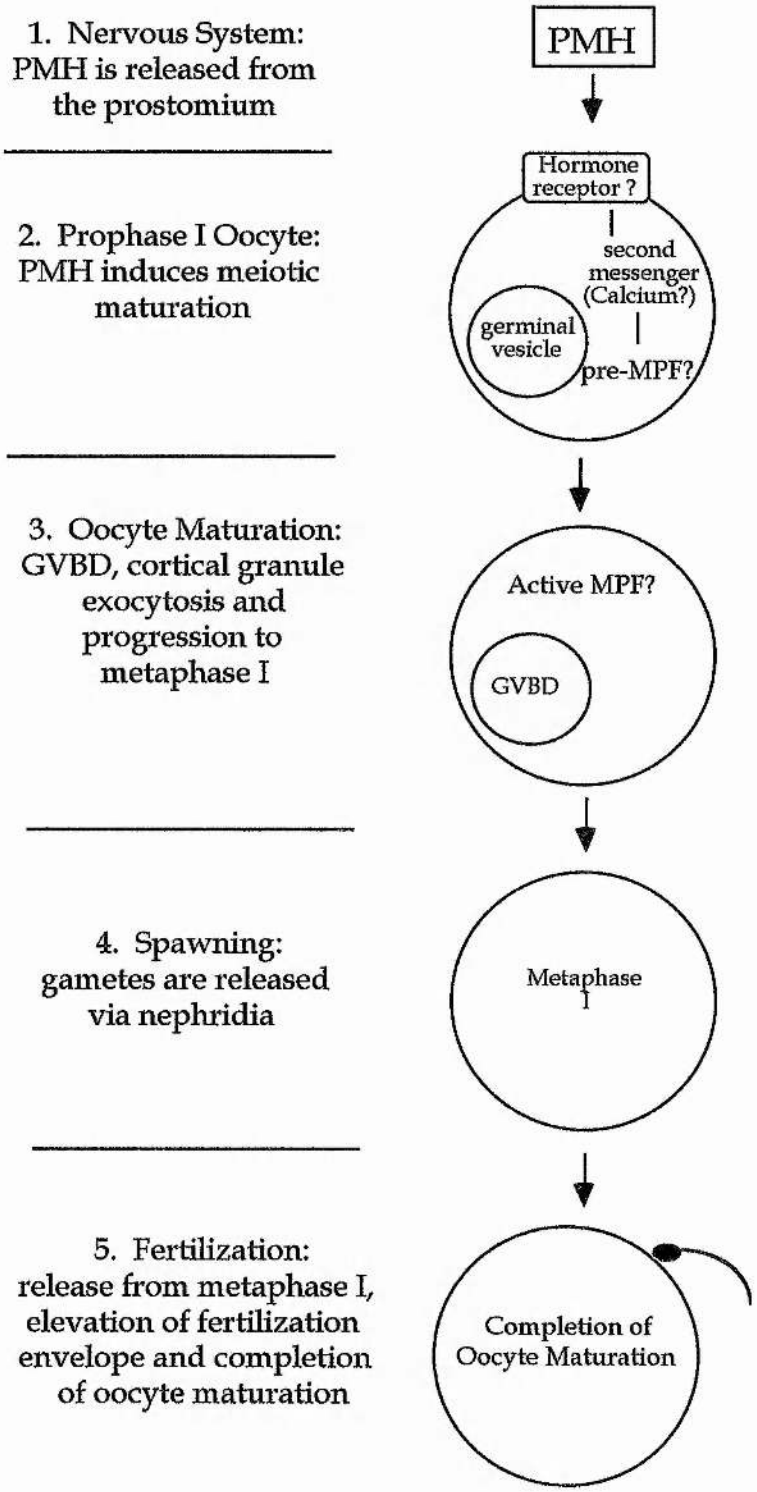


Figure 8.2: Oocyte maturation in *Arenicola defodiens*

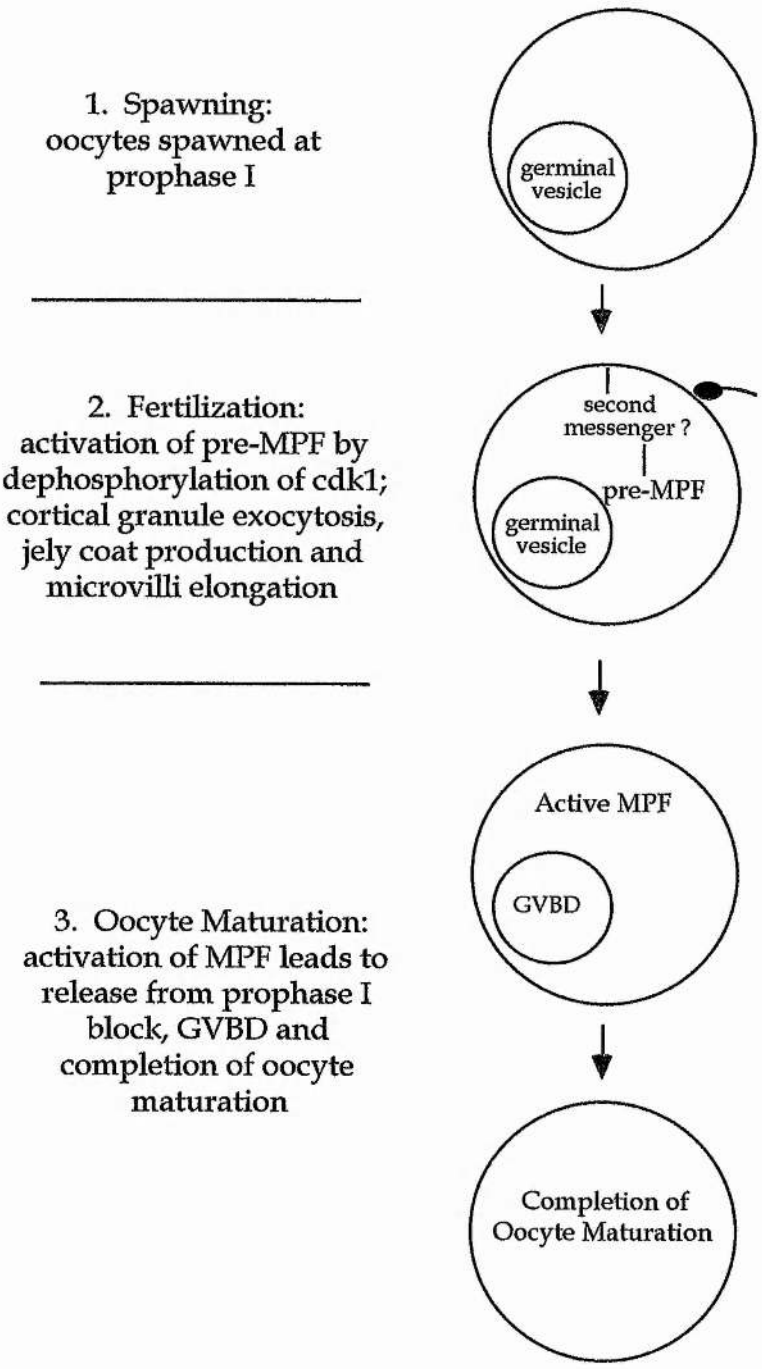


Figure 8.3: Oocyte maturation in *Nereis virens*

- Does the acquisition of fertilizability coincide with the development of the calcium release mechanisms (discussed in section 5.4)?

Search for the Second Messenger

Several avenues have yet to be investigated in the search for the second messenger during CMF induced meiotic maturation in *Arenicola marina*. Evidence shows that the prophase I block in many organisms is maintained by elevated cAMP levels (section 1.3.4). However, it has also been found that a reduction in cAMP facilities meiotic maturation but, alone, is not sufficient to induce it (Meijer *et al.*, 1989b). The hunt for the identity of the second messenger has not been firmly established in any organism and further research is required. In addition to further exploration into the role of cAMP, more work should be directed towards the role of protein kinase C, and the possibility of inhibitory proteins that prevents progression from prophase I. Evidence for this was gained from oocytes of the *Chaetopterus pergamentaceus*. The prophase I oocytes of this polychaete will mature in response to incubation with cyclohexamide, which inhibits protein synthesis (Zampetti-Bosseler *et al.*, 1973). It is possible that cyclohexamide prevents the synthesis of inhibitory proteins and thereby allows meiotic progression.

Chemical induction of meiotic reinitiation is one useful method in which to determine the potential pathways involved during oocyte maturation. A number of specific chemicals have been shown to induce meiotic maturation, such as calcium in echinoderm oocytes (Moreau *et al.*, 1978) but further investigation reveals these cations are acting via a different signalling transduction pathway from the one evoked during *in vivo* meiotic reinitiation (Kikuyama and Hiramoto, 1991; Stricker *et al.*, 1994). The oocytes of *A. marina* are unique in that no chemicals have so far been found that induce the release of the prophase I block. Yet, because prophase oocyte activation in *A. marina* is not susceptible to many chemical agents

or spontaneous maturation, it is potentially an excellent model with which to search for the true native second messenger during hormone induced oocyte maturation.

MPF

Studies on MPF regulation in the oocytes of *Arenicola marina* and *Nereis virens* have revealed two novel regulation systems, not hitherto described. In addition to the pool of cdk1/cyclin B activated during the prophase to metaphase transition, *A. marina* oocytes contained a second, larger, pool of cdk1 not utilised during meiotic maturation. Further work should be carried out to determine the function of this second large pool of cdk1. Initial studies should be directed towards cdk1 and cyclin B at insemination following the release of the metaphase I block to discover if this stockpile of cdk1 is used to complete meiotic maturation or for use in mitotic divisions during embryogenesis. In addition, research should be carried out to characterise MPF activation and regulation in *A. defodiens* oocytes for comparison.

As the results indicated, *Nereis virens* oocyte MPF regulation shows more similarities with fission and budding yeast, than to eukaryotes, in that only the tyrosine 15 residue (or equivalent to) is phosphorylated when inactive. Future work should be directed towards discovering the effect of various phosphatases, such as pyp3, on pre-MPF from *N. virens* oocytes. Pyp3 dephosphorylates the tyrosine 15 residue only (Millar *et al.*, 1992; Borgne and Meijer, 1996). If full activation of *N. virens* pre-MPF occurs after incubation in this phosphatase, this would support the hypothesis that only tyrosine 15 is phosphorylated. This work should be followed by phosphoamino acid analysis and peptide mapping to determine the actual sites of phosphorylation. If the cdk1 subunit in pre-MPF is indeed phosphorylated at tyrosine only, this would provide an excellent model

system with which to study the control of tyrosine phosphorylation and dephosphorylation. Investigation of the regulation and control of the individual residues is more difficult in the MPF of other organisms, due to the dual-residue inhibition (Krek and Nigg, 1991; Norbury *et al.*, 1991). For example, using mammalian MPF to study the individual regulation of each residue, requires the one residue to be substituted with a different amino acid (Norbury *et al.*, 1991).

Hormone Regulation of Oocyte Maturation and Spawning

Investigation of other marine invertebrates reveals that oocyte maturation and spawning of species belonging to the same phylogenetic class is controlled by the same the molecular signal(s) for gamete maturation and spawning. For example, oocyte maturation and spawning is regulated by 5-HT in all bivalves molluscs, whereas gonad stimulating hormone (GSH), followed by 1-methyladenine (1-MeAde) operates in all asteroid echinoderms (see section 1.2). Oocyte maturation has been investigated in several polychaetes and each species has been found to be regulated by a different mechanism, including the two very closely related species *Arenicola marina* and *A. defodiens*. Furthermore, in all other invertebrates investigated, the molecular signal(s) for gamete maturation and spawning are interchangeable between males and females: starfish (Meijer and Guerrier, 1984); molluscs (Hirai *et al.*, 1988; Ram *et al.*, 1993) and the polychaete *Pectinaria gouldii* (Tweedell, 1980). The sex-specificity with respect to the hormonal cascade in control of gamete maturation and spawning in *Arenicola marina* is unique and certainly warrants further investigation. This should include further chemical characterisation of PMH in both *Arenicola marina* and *A. defodiens*. Initial experiments should be directed towards determining the chemical nature of PMH. For example, to study the activity of PMH after trypsin treatment and boiling, to determine if it is a peptide-like. In addition,

further investigations should be carried out on the purification and characterisation of CMF and how the release of PMH triggers the activation of CMF.

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